# The Journal of Comparative Pathology and Therapeutics

Vol. 57. No. 4. OCTOBER, 1947. Price 8 shillings and sixpence.

1. The columns of the Journal are open for contributions recording original research in veterinary science.

2. The Editorial Board accepts papers bearing on the above subject on the understanding that they are not being offered elsewhere for publication. It reserves the right to reject any papers submitted to it for publication.

3. It is essential that all articles should be concise. Any necessary allusions to matter already published should be brief and limited strictly to those relevant to the satisfactory exposition of the authors' own work.

4. Citation of literature in the text should be accompanied by the year of publication. Authors' names (followed by year, publication, etc.) should be arranged alphabetically in a list at the end of the paper.

5. Papers for publication, exchange journals, etc., should be sent to Prof. T. J. Bosworth, "The Beeches," Streatley, Berks, or to any member of the Editorial Board.

6. Correspondence concerning business matters should be addressed to the Publishers, Messrs. H. R. Grubb, Limited, Poplar Walk, Croydon, Surrey.

7. The net price of each volume of four numbers is £1 10s., paid in advance to the publishers. Single numbers may be obtained at a price of 8s. 6d. each.

Additional copies and reprints ordered before the issue is printed may be obtained from the publishers.

## CONTENTS

PAGE

Studies on the Source of the Staphylococcal Infection Found in Tick Pyaemia in Lambs. By A. Foggie ... 245

An Immunological Study of *Pasteurella septica*. By R. S. Roberts ... ... ... ... ... ... 261

Studies on *C. pyogenes* Antitoxin in Relation to Immunity :—

I. The Production of *C. pyogenes* Antitoxin in the Serum of Normal Dairy Cows following the Inoculation of Alum-Precipitated Toxoid and of a Commercial Toxoid. By B. Weitz ... 279

II. The Effect of Alum-Precipitated Toxoid and Vaccine on the Artificial Infection of the Udder of the Ewe with *C. pyogenes*. By B. Weitz and R. Langridge ... ... ... ... ... 286

The Maintenance of Cattle under Conditions of Tsetse-Fly Infestation in the Field. By J. K. H. Wilde ... 294

A Study of the Re-exposure to *Tr. foetus* of Animals already Exposed to the Infection as Virgin Heifers with Some Observations on the Localization of Antibody in the Genital Tract. By W. R. Kerr and Muriel Robertson ... ... ... ... ... ... 301

Iron Metabolism in Piglet Anaemia. By J. A. J. Venn, R. A. McCance and E. M. Widdowson ... ... ... 314

# STUDIES ON THE SOURCE OF THE STAPHYLOCOCCAL INFECTION FOUND IN TICK PYAEMIA OF LAMBS

By

Angus Foggie

*Animal Diseases Research Association, Moredun Institute, Gilmerton, Midlothian.*

## INTRODUCTION

Although tick pyaemia, first described by M'Fadyean (1894), has been associated by him and all subsequent workers with tick infestation, there has been no published work on the source of the staphylococcal infection encountered in lambs suffering from this disease. The present paper describes experiments designed to show the probable source of the infection.

A consideration of the rôle played by the tick shows the following possibilities.

The tick may act as a true vector of the disease. If this be the case, a staphylococcal infection acquired by the tick while feeding on a lamb suffering from pyaemia, is retained in the tick's body through the moulting period to the next instar. This internal infection is transmitted to a lamb in the following season when the tick engorges.

The cuticle of the tick may carry the infection or the skin of the lamb may be infected ; in either case the staphylococci gain entrance to the tissues of the lamb by means of the wound caused by the tick while feeding.

An attack of another tick-borne disease such as louping-ill or tick-borne fever may so lower the lamb's resistance to infection that an invasion of the bloodstream by staphylococci occurs. In this case the mode of entry of the organism might not necessarily be through the wound caused by tick bite.

## The Tick as a Vector of Tick Pyaemia

In order to test the first hypothesis it was necessary (*a*) to find a means of sterilising the cuticle without causing injury to the tick ; (*b*) to produce an internal infection of the tick by feeding it on a host suffering from a staphylococcal septicaemia ; (*c*) to demonstrate that internal infection of the tick had occurred ; and (*d*) to maintain suitably infected live ticks and examine them at intervals to determine whether the staphylococci within the tick remained viable until the tick had reached the next instar and was ready to attach and feed on a fresh host. If this were demonstrated and if the disease could be reproduced by feeding such experimentally infected ticks on lambs, the rôle of the tick as a true vector would be established.

Engorged females of *Ixodes ricinus* were collected from sheep and the stock maintained in the laboratory by allowing the instars,

which were not required for experiment, to engorge on hedgehogs. The progeny of these ticks were used in the following experiments.

### THE STERILISATION OF THE CUTICLE OF THE TICK

Cetavlon has been shown to be an effective agent for the sterilisation of human skin (Williams *et al.*, 1943, 1944). These workers found that in a dilution of 1 : 218,700, it inhibits the growth of *Staphylococcus aureus* after incubation for 18 hours and that in a culture which originally gave a colony count of 130,000, eight minutes' exposure to a 1 : 10,000 concentration of Cetavlon reduced the count to 12.

Staphylococci isolated from a case of tick pyaemia when suspended in a 1 : 256,000 solution of Cetavlon for 30 minutes followed by three washings in sterile saline solution, failed to grow on inoculation into nutrient broth.

Engorged ticks of all three instars, washed in a 1 per cent. solution of Cetavlon for 30 minutes followed by three rinsings in sterile water, have been observed to complete the next stage of their life-cycle. The available data suggests that the mortality in ticks so treated is no greater than in untreated ticks. An engorged larval tick maintained in a 1 per cent. solution of Cetavlon remained alive for six weeks. A solution of this strength, therefore, is non-toxic or of low toxicity for ticks.

Thirty engorged larvae were washed in water ; ten of them were ground up in a mortar with 5 c.c. of saline and 0·2 c.c. of the resulting emulsion was plated on sheep blood agar. No growth of staphylococci was observed. The remaining 20 ticks were transferred for 30 minutes to a No. 8 Brown's scale suspension of *Staph. aureus,* and were then washed in 25 c.c. of distilled water. When ten of the larvae were ground and plated as above, 0·2 c.c. of emulsion gave a growth of 150 colonies of *Staph. aureus.* The remaining ten larvae were transferred to a 1 per cent. solution of Cetavlon for 30 minutes, washed three times in distilled water, ground up and plated. No growth of staphylococci was observed.

This test shows that the cuticle of ticks contaminated by a culture of staphylococci may be effectively sterilised by washing for 30 minutes in a 1 per cent.solution of Cetavlon.

### THE PRODUCTION OF AN INTERNAL STAPHYLOCOCCAL INFECTION IN EXPERIMENTALLY FED TICKS

The method of obtaining infected ticks was as follows : A batch of unengorged larvae or nymphs was liberated in a cloth bag into which a guinea pig was placed and left overnight to allow time for the ticks to attach. The following morning the guinea pig was tranferred to a cage with open wire flooring standing over a tray containing water. As the ticks engorged and dropped off they were collected from the water. On the second or third day after the

ticks had been applied to the guinea pig the latter was given an intracardial injection of a suspension of *Staph. aureus* sufficient to produce a septicaemia lasting from three to six days (McDiarmid, 1946), using an inoculum of 0·05 c.c. of a No. 8 Brown's scale suspension of either strain 6591 or strain 1468, made up to a volume of 0·5 c.c. Strain 6591 was isolated from the mouth of a ewe ; strain 1468 is the same strain after passage through a guinea pig. They appear to be identical in virulence and other characteristics. The mean period of survival of 21 guinea pigs after inoculation with this dose, was 4·1 days ± 1·83 days. As larval and nymphal ticks take from three to seven days to engorge it was thus possible to ensure that the majority of the ticks completed their engorgement after the guinea pig had been infected. The engorged ticks were collected daily and a sample of each collection was examined for the presence of staphylococcal infection.

In early experiments groups of ten ticks, washed in Cetavlon and then in sterile water as described above, were ground up in 5 c.c. of sterile saline, 0·2 c.c. of the suspension was plated on blood agar and a count of the colonies was made. As the numbers of staphylococci in different samples from the same collection of larvae were found to vary greatly, in later experiments individual ticks instead of composite samples were examined bacteriologically. The engorged ticks were treated as before, but each tick was ground up with sterile precautions on a glass plate. The resulting smear was emulsified in a loopful of sterile saline and stroked on to a sheep blood agar plate which was examined for staphylococci.

### THE DEMONSTRATION OF INTERNAL INFECTIONS IN EXPERIMENTALLY FED TICKS

A group of 399 ticks from eight batches of engorged larvae that had been fed on infected guinea pigs were examined individually at the time of their collection ; 176 of them were found to be infected internally with staphylococci.

The mean of the percentages of infected larvae from these eight experiments is 38 per cent. with a standard deviation of ± 20 per cent.

### THE SURVIVAL OF STAPHYLOCOCCAL INFECTION IN EXPERIMENTALLY FED TICKS

Other ticks from the same batches of larvae were allowed to develop and were examined from one to eight months after engorgement, but none showed the presence of staphylococci. Table I gives the results of the examination of a group of larvae obtained from one of the infected guinea pigs mentioned above.

Besides the ticks examined bacteriologically, 116 nymphs were obtained from this batch of larvae. In June, 1945, seven months after collection, they were washed in Cetavlon and in water, and

TABLE I

| *Date* | *Time* | *Number of larvae collected* | *Number of larvae examined* | *Number of larvae infected* |
|---|---|---|---|---|
| 2.10.44 | 3 p.m. | Larvae applied to guinea pig | | |
| 5.10.44 | 10 a.m. | 3 | 3 | 0 |
| | 4 p.m. | 109 | 24 | 2* |
| 6.10.44 | 10 a.m. | 32 | 10 | 0 |
| | 12 noon | Guinea-pig infected | | |
| | 4 p.m. | 114 | 24 | 12 |
| 7.10.44 | 10 a.m. | 25 | 12 | 10 |
| 8.10.44 | 12 noon | 112 | 24 | 14 |
| 9.10.44 | 10 a.m. | 27 | 12 | 4 |
| | 2 p.m. | Guinea-pig killed *in extremis* | | |
| Totals after infection | | 278 | 72 | 40 |

* The staphylococcal infection in these larvae is an example of a naturally-occurring skin infection being picked up by the larvae during engorgement. Other instances of this phenomenon were encountered in experiments on the effect of feeding ticks on contaminated skin.

examined for the presence of staphylococci but none was found to be infected.

The ticks in these experiments had been maintained at a temperature of 24° C. in order to hasten the moulting process. It appeared possible that this temperature, which is abnormally high compared with field conditions, was responsible for the failure of the staphylococci to survive the moulting period. A batch of larvae after engorging on an infected guinea pig was, therefore, retained in the cold store at a temperature of 4° C. Samples of these larvae were examined periodically for staphylococcal infection. The results of this experiment are shown in Table II.

TABLE II

| *Date* | *Number of larvae collected* | *Number of larvae examined* | *Number of larvae infected* |
|---|---|---|---|
| 20.8.45 | Larvae applied to guinea-pig | | |
| 22.8.45 | 252 | 20 | 0 |
| | Guinea-pig infected | | |
| 23.8.45 | 61 | 10 | 6 |
| 24.8.45 | 238 | 39 | 18 |
| 25.8.45 | 15 | 4 | 4 |
| | Guinea-pig dead | | |

Examination of samples of larvae taken at intervals from the batch of larvae collected on August 24th gave the results seen in Table III.

It thus appears that a staphylococcal infection persists for less than three months in the body of the engorged larval tick even when the larvae are kept at low temperatures. It is, therefore, unlikely

that, under field conditions, larval ticks which become infected by feeding on lambs suffering from a staphylococcal septicaemia will retain the infection into the next instar. According to Campbell (unpublished) ticks under natural conditions usually take a year to complete each instar.

A much smaller proportion of the number of nymphs applied to guinea pigs became attached and engorged, and smaller numbers of engorged nymphs were available for experiment. Eleven out of

TABLE III

| *Date* ... ... ... | 24.8.45 | 22.9.45 | 1.11.45 | 30.11.45 | 11.1.46 |
|---|---|---|---|---|---|
| Number of larvae examined | 39 | 20 | 20 | 21 | 20 |
| Number of larvae infected | 18 | 3 | 1 | 0 | 0 |

41 engorged nymphs, collected off infected guinea pigs were found to have an internal staphylococcal infection. Fifty-three of the remaining nymphs from these batches were examined at intervals of from one to five months after engorgement, but none was infected. It would thus appear that a varying percentage of nymphs, fed on guinea pigs suffering from a staphylococcal septicaemia, become infected, but, as in the case of larvae, this infection is not retained through the moult to the following instar.

### THE EXAMINATION OF TICKS FROM PYAEMIA CASES

Engorged ticks collected from cases of tick pyaemia are occasionally found to be infected with staphylococci, but the incidence is not high. Of a series of 19 engorged females and five males collected from cases only one was found to be infected.

### THE EXAMINATION OF TICKS FROM PASTURE

Unengorged ticks, collected by dragging a blanket over the pasture on farms where tick pyaemia is prevalent, were examined for staphylococci by washing in Cetavlon and plating individually as described above. In all, 362 nymphs, five females and five males were examined from this source. None of them was found to be infected.

The above experiments are strong evidence that the tick does not act as a true vector of tick pyaemia and that if tick-bite is the mode of entry of the staphylococcus into the lamb's body the tick merely acts as an inoculator of an infection derived from some source outside its own body.

## THE TICK AS AN INOCULATOR OF EXTERNAL INFECTION

As the most probable source of the causal organism of tick

pyaemia appeared to be a skin infection of the lamb, experiments were designed to determine whether skin infections of staphylococci occur in lambs and whether the organisms present are similar to those isolated from cases of tick pyaemia.

### THE DETERMINATION OF STAPHYLOCOCCAL INFECTION IN EWES AND LAMBS BY THE EXAMINATION OF SWABS

A study was made of infections of haemolytic staphylococci in the natural orifices and skins of ewes before lambing and on the skin of their lambs after birth.

*Animals.* A flock of 19 Blackface ewes was purchased from a tick-free hill farm and kept at Moredun. Swabs were taken from these ewes on the following dates : November, 1943, 24.1.44, 21.2.44, 21.3.44, 4.4.44, 19.4.44, 2.5.44, 15.5.44, and within 24 hours of lambing. Lambing started on March 19th and finished on May 31st. Skin swabs were taken from the lambs shortly after birth and at intervals of 14 days thereafter.

Twenty-four Cheviot ewes on a tick-infested farm at Kirkconnel, Dumfriesshire, where pyaemia occurs, were ear-tagged and swabs were taken on the following dates : 2.2.44, 28.2.44, 27.3.44, 12.4.44, 24.4.44, 8.5.44 and 24.5.44. Lambing started about April 15th and finished about May 15th. Skin swabs were taken from the lambs at intervals of 14 days from within 13 days of birth until June 5th.

*Methods.* The following method of taking swabs was carried out in all cases :

*Ewes.* Mouth : a sterile swab was passed into the mouth, between the molar teeth and the cheek, and withdrawn. Nose : a sterile swab was passed up the left nostril and withdrawn. Skin of udder : a sterile swab was dipped in sterile saline solution and rubbed over an area about 1 inch square on the surface of the udder. It was then passed over the point of one teat. Skin of thigh : a sterile swab moistened with saline was rubbed on the inner surface of the thigh. Vagina : the lips of the vulva were opened and a sterile swab was passed into the vagina and withdrawn.

*Lambs.* Four sterile swabs moistened with saline were rubbed on the hairless skin of the axillae and insides of the thighs.

*Examination of swabs.* The swab was transferred to 5 c.c. of Hartley broth, allowed to soak at room temperature for approximately two hours, and then thoroughly shaken in the broth. A series of dilutions decreasing by 1 : 10 in saline having been made, 0·2 c.c. of the broth and 0·2 c.c. of each dilution were sown on to a series of 5 per cent. sheep blood agar plates which were incubated overnight, allowed to cool for one hour at room temperature and the colonies of haemolytic staphylococci counted. Cooling after incubation facilitates the identification of the $\alpha\beta$ haemolysis shown by the majority of the staphylococci encountered.

The weight of infection in positive swabs, as given in the tables, is estimated from the number of colonies obtained from plating 0·2 c.c. of the Hartley broth. This estimate was arrived at by multiplying the number of staphylococcus colonies on each plate of a series by its respective dilution and taking the mean of the answers.

## RESULTS

The results are set out in Tables IV and V.

*Ewes.* A study of these tables indicates that there was a tendency for more ewes on both farms to carry staphylococcal infections during the winter months than in spring. The number of heavy infections is also greater in winter than in spring. At lambing five of the 17 Moredun ewes which lambed, were infected ; and at the first swabbing after lambing, 13 of the 22 Kirkconnel ewes, which lambed, were infected. This difference is not significant ($\chi^2$ = 3·399, P = 0·1 to 0·05). None of the infections at the first swabbing after lambing was heavy. The figure A300 in ewe 851 is the count of staphylococci from an abscess following tick-bite and is omitted from the above comparisons. As is shown in the tables, when infections were detected in individual ewes on several different occasions they were usually of the same bacteriophage type.

*Lambs.* Seven out of 19 lambs born at Moredun and ten out of 22 lambs from ear-tagged ewes at Kirkconnel showed a naturally occurring skin infection. This difference in incidence is not significant ($\chi^2$ = 0·311, P = 0·7 to 0·5). The staphylococcal infections in tick abscesses (lambs Nos. 461 and 531) are again omitted from this comparision. Swabs were taken from several other tick abscessess but these failed to show staphylococcal infection and are not recorded.

Table VI gives the distribution of infection in lambs in relation to infection in their dams at lambing.

TABLE VI

| *Farm* | *Ewe not infected* | | *Ewe infected* | |
|---|---|---|---|---|
| | *Lamb not infected* | *Lamb infected* | *Lamb not infected* | *Lamb infected* |
| Kirkconnel | 6 | 4 | 6 | 6 |
| Moredun | 8 | 5 | 4 | 2 |
| Totals | 14 | 9 | 10 | 8 |

There is no correlation between the presence or absence of infec tion in the ewes and in their lambs ($\chi^2$ = 0·118, P = 0·8 to 0·7). If, however, the 'phage types isolated from lambs are compared with those isolated from their mothers it is found that on 18 occasions the type present on the lamb's skin was the same as that isolated

TABLE

EXAMINATION OF SWABS FROM

| *No. of ewe* | *Ewes* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Nov.*, 1943 | 24.1.44 | 21.2.44 | 21.3.44 | 4.4.44 | 19.4.44 | 2.5.44 | 15.5.44 |
| 649 | N 11 88 | N 8 UT | M 7 X2 | – | – | – | – | – |
| 650 | N 7 X2 | N 8 X2 | N 1 UTB | M 1 UTB X2 | M 12 UTB | N 1 UTB | – | – |
| 652 | – | M 8 S7 | M 54 S7 | M 13 X2 | – | – | M 16 | – |
| 653 | – | M 5 X2 | – | – | – | – | – | – |
| 654 | N 15 X2 | – | M 6 | M 6 UT | M 4 | N 1 X2 | – | – |
| 655 | – | M 1 | – | – | – | – | – | * |
| 656 | V 36,000 X2 | V 20 X2 | M 27 X2 | – | – | – | M 100 X2 U 3 X2 | – |
| 658 | – | V 700 X2 | V 900 X2 | V 1 X2 | – | – | – | – |
| 659 | M 13 X2 N 50 | M 15 X2 | – | V 1 X2 | M 2 X2 | M 1 X2 T 1 X2 | M 2 X2 | M 31 |
| 660 | – | – | – | – | – | M 1 UT | M 49 UT | M 12 |
| 661 | N 1,500 X2 V 100,000 X2 | M 1 88 | N 1 X2 | M 6 X2 | – | – | U 80 X2 | – |
| 662 | M 40 X2 | M 1 X2 | – | – | – | – | M 1 X2 | M 1 |
| 664 | – | – | – | – | – | – | – | – |
| 665 | N 50,000 X2 U 1,300 X2 | N 70 X2 M 5 X2 | N 380 X2 M 2 X2 | – | – | – | – | – |
| 666 | – | – | – | – | M 1 UT | – | – | – |
| 668 | – | N 1 X2 | – | – | – | – | – | – |
| 670 | – | – | M 5 88 | – | – | – | M 10 X2 | M 24 |
| 671 | M 100 S7 | – | M 3 X2 | M 50 | – | M 3 X2 | M 100 | M 11 |
| 672 | – | M 60 X2 N 4,000 X2 | M 13 X2 N 8 X2 | – | M 100 X2 N 2 X2 | M 60 X2 N 15 X2 | M 100 X2 | M 5 |

*Abbreviations*:
M = mouth.
N = nose.
U = skin of udder.
T = „ thigh.
V = vagina.
Number = number of haemolytic staphylococci in 0·2 c.c. broth.

IV

EWES AND LAMBS AT MOREDUN

| *Date of lambing* | *Ewe at lambing* | *Lamb at birth* | *No. of lamb* | *Lambs* | | | |
|---|---|---|---|---|---|---|---|
| | | | | 4.4.44 | 19.4.44 | 2.5.44 | 15.5.44 |
| 3.5.44 | – | – | 756 | * | * | * | – |
| 11.4.44 | – | – | Died | 743 twinned from ewe 659 * | – | – | – |
| 11.4.44 | – | – | 747 | * | LT 1 X2 | Expt. | – |
| 12.5.44 | – | – | 765 | * | * | * | – |
| 19.3.44 | M 6 | – | 739 | – | – | – | – |
| 26.3.44 | – | – | 740<br>741 | –<br>– | –<br>LA 7 X2 | –<br>– | –<br>– |
| 11.4.44 | M 1 | – | 746 | – | LT 7 X2 | Expt. | – |
| 31.5.44 | – | – | 768 | * | * | * | * |
| 10.4.44 | T 1 | – | 743<br>744 | To ewe 650<br>* | – | Expt. | Expt. |
| 13.5.44 | M 12 | LT 1UT | 766 | * | * | * | Lt. 1 UT |
| 10.4.44 | – | – | 745 | * | RT 1 X2 | – | Expt. |
| 31.3.44 | – | – | 742 | – | LT 1 X2 | – | – |
| 13.5.44 | – | – | 767 | * | * | * | – |
| 16.4.44 | – | – | 750 | * | – | RT 2 | – |
| 16.4.44 | – | – | 751 | * | – | – | – |
| 3.5.44 | – | – | 758 | * | * | – | – |
| | Not in lamb | | | | | | |
| 15.4.44 | M 3 | – | 748<br>749 | *<br>* | –<br>– | –<br>– | –<br>– |
| | Not in lamb | | | | | | |

RA = right axilla.
LA = left axilla.
RT = right thigh.
LT = left „
Expt. = experimental infection.
– = no staphylococci detected.
* = animal not examined.

X2 = phage type.
88 = „
S7 = „
UTA = „
UTB = „
UT = untypeable.

TABLE
EXAMINATION OF SWABS FROM

| *No. of ewe* | *Ewes* | | | | | | |
|---|---|---|---|---|---|---|---|
| | 2.2.44 | 28.2.44 | 27.3.44 | 12.4.44 | 24.4.44 | 8.5.44 | 24.5.44 |
| 832 | – | – | – | – | M 1 X2 | †– | * |
| 833 | M 110 UTA<br>N 3,000 UTA | – | M 1 UTA | – | – | †– | * |
| 834 | M 30 X2<br>N 3,300 S7 | M 50 S7<br>N 10 S7 | Ewe died | | | | |
| 835 | – | – | – | – | – | †– | * |
| 836 | – | – | – | – | M 1 X2 | †– | * |
| 837 | M 18 X2 | M 1 X2 | – | M 15 X2 | M 3 X2 | †M 6 | * |
| 838 | M 13 88<br>M 53 88 | – | – | – | †M 7 88 | – | * |
| 839 | M 1 88<br>N 21 88 | – | – | M 1 88 | – | – | †– |
| 840 | M 8 X2<br>N 350 X2 | – | M 2 X2 | – | M 18 X2 | †M 20 X2 | * |
| 841 | M 13 88<br>N 1 88 | M 2 88 | M 4 UTA | – | M 3 88 | – | Not in lamb |
| 842 | M 12 X2 | – | M 1 X2 | – | †– | – | * |
| 843 | M 25<br>N 300 X2<br>U 1 UT | M 12 X2<br>N 80 X2 | M 2 X2 | – | M 3 X2 | M 9 | †M 7 X2 |
| 844 | N 7 UTA | – | – | – | †M 2 UTA | M 6 X2 | M 1 |
| 845 | M 45 X2<br>N 10,000<br>V 2 X2 | N 1 X2<br>V 163 X2 | M 24 X2<br>V 900 X2 | M 8 X2 | – | M 21 X2<br>N 1 | †N 1 X2<br>V 1 X2 |
| 846 | – | – | – | – | – | †– | * |
| 847 | M 400 X2<br>N 11 X2 | – | M 1 X2 | M 14 X2 | – | †M 5 X2 | * |
| 848 | M 10 UTA | M 2 UTA | M 5 UTA | – | N 1 UTA | †– | * |
| 850 | – | – | – | – | – | – | †N 2 |
| 851 | V 10 88 | – | – | – | – | †A 300 88 | * |
| 852 | M 42 UTB<br>N 2 UTB<br>V 87 88 | M 4 UTB | M 10 | – | V 2 88 | M 13<br>V 15 | †U 7 X2<br>V 6 88 |
| 853 | M 280 88<br>N 53 88<br>V 2 UTA | V 6 UTA | – | – | M 3 88<br>V 1 | †V 1 UTA | – |
| 854 | – | – | – | – | †M 1 | – | * |
| 855 | M 2<br>V 41 | – | – | M 1 X2 | – | †M 6 X2 | * |
| 856 | – | – | – | M 3 UTA | M 8 | M 196 M | †M 26 UTA |

*Abbreviations*: As in Table VI.

V
EWES AND LAMBS AT KIRKCONNEL

| *No. of lamb* | *Lambs* | | | | |
|---|---|---|---|---|---|
| | 24.4.44 | 8.5.44 | 24.5.44 | 5.6.44 | 19.6.44 |
| 464 | * | – | RA 1 S7 | – | * |
| 462 | * | – | – | – | * |
| 461 | * | – | Escaped during swabbing | | A 20 S7 |
| 465 | * | – | RT 3 X2 | – | * |
| 526 | – | – | RA 1 X2 | – | * |
| 458 | * | – | – | – | * |
| 894 | * | * | – | * | * |
| 459 | * | – | – | – | * |
| 437 | RA 1 X2 | – | – | RA 50 X2<br>LA 2 X2 | * |
| 994 | * | * | – | RA 10 X2<br>LA 1 X2<br>RT 1<br>LT 1 S7 | * |
| 468 | LT 2 UTA | LA 3 | LA 1<br>RT 2 88<br>LT 78 S7 | A 1 X2<br>RA 1 X2<br>LT 1 X2 | * |
| 77 | * | * | – | – | * |
| 532 | * | – | – | – | * |
| 463 | * | – | – | – | * |
| 466 | * | – | – | – | * |
| 992 | * | – | – | – | * |
| 531 | * | * | – | A 300 X2 | – |
| 993 | * | * | – | LA 1 X2 | * |
| 460 | * | – | RA 1<br>LA 9 UTA | – | * |
| 438 | LA 10 | – | – | – | – |
| 467 | * | – | RA 3 UTA<br>LA 15 88 | LA 1 88 | * |
| 991 | * | * | – | – | * |

A = tick abscess. † = first swabbing after lambing.

from the mother, while on seven occasions the types were different. Unfortunately, type X2 is of such common occurence that the exact source of an infection with this type cannot be definitely decided.

None of the lambs in this experiment developed pyaemia.

### THE INCIDENCE OF SKIN INFECTIONS IN TICK PYAEMIA CASES COMPARED WITH THAT IN NORMAL LAMBS

The incidence of staphylococcal infections in skin swabs from 19 cases of tick pyaemia was compared with the incidence in those from 87 normal lambs of susceptible age on tick-infested farms with the results shown in Table VII.

Table VII

| *Lambs* | *Skin infected* | *Skin not infected* | *Totals* |
|---|---|---|---|
| Pyaemia cases ... | 9 | 10 | 19 |
| Normal lambs ... | 20 | 67 | 87 |
| Totals ... | 29 | 77 | 106 |

$\chi^2 = 4{\cdot}664$. $P = 0{\cdot}05$ to $0{\cdot}02$.

There appears to be a significantly greater number of skin infections in lambs affected with pyaemia than in lambs not so affected.

These experiments show that infections of haemolytic staphylococci occur in the natural orifices and on the skin of ewes and also on the skin of lambs. The infections on lambs' skins may be derived from their mothers but infections from other sources may also occur. There appeared to be little difference in the incidence of the infections in a tick-free and a tick-infested farm. Skin infections are more prevalent in lambs suffering from pyaemia than in normal lambs.

### A COMPARISON OF HAEMOLYTIC STAPHYLOCOCCI FROM EXTERNAL INFECTIONS IN EWES AND LAMBS AND THE HAEMOLYTIC STAPHYLOCOCCI FROM TICK PYAEMIA CASES

A bacteriological comparison of 211 strains of haemolytic staphylococci obtained from ewes and lambs, as described in the previous section, and 56 strains of staphylococci isolated from cases of tick pyaemia in lambs was carried out.

*Methods.* During 1944 the strains, after isolation, were sown on to Hartley agar slopes in McCartney bottles, incubated overnight and retained at room temperature until examined. In 1945 Worth's medium was substituted for Hartley agar. The seeded cultures were not incubated but were stored at room temperature. Before examination a sub-culture was made on to Hartley agar and incubated for 18 hours. The growth was washed off into 2 or 3 c.c. of sterile saline and used for the following tests.

*Pigment production.* Loopfuls of culture suspension were stroked on to plates of a medium containing 33 per cent. sterile milk added to Hartley agar. The plates were incubated at 37° C. for 48 hours and stored at room temperature for five days.

Three classes of pigment were observed. Golden : a deep orange-golden colour. Fawn : this class, which contains most of the strains recovered from sheep, ranges from a brownish-golden through fawns of diminishing intensity to just off-white colours. Considerable difficulty was experienced in differentiating between golden and the brownish-golden at one end of the range, and between white and off-white at the other. White : china-white.

*Haemolysis.* Haemolysin production was examined by the method of Bryce and Rowntree (1936) using a loopful of the culture suspension sown as a single drop on a 5 per cent. sheep blood agar plate, incubated for 18 hours and allowed to cool at room temperature for two hours. An attempt was made to gauge the degree of haemolysin production by measuring the widths of the clear ($\alpha$) zone and the partial ($\beta$) zone of haemolysis, but these measurements were found to vary greatly with the same strain from plate to plate and also on different parts of the same plate.

*Coagulase production.* Citrated rabbit plasma was diluted 1 : 5 with normal saline and 0·5 c.c. of the dilution was pipetted into test-tubes of 8 m.m. diameter. Each tube was inoculated with a loopful of the different culture suspensions under examination. On each occasion controls containing diluted rabbit plasma only were included. The tubes were incubated for three hours, examined for coagulation, and allowed to stand on the bench overnight. Positive coagulation was recorded if, at either of the readings, the tube could be inverted without loss of fluid.

*Pathogenicity for mice.* A suspension equal in opacity to No. 8 of the Brown's scale, from each culture to be tested, was diluted 1 : 3 and 0·3 c.c. was injected intravenously into three mice. Deaths were recorded for eight days after inoculation. When one or more mice survived the inoculation, the test on that strain was repeated. A positive result was recorded when more than half of the mice inoculated with the strain under test died within eight days.

## RESULTS

The results of these tests are set out in Table VIII which shows that all the strains of haemolytic staphylococci from external infections in ewes and lambs and from pyaemia cases are coagulase-positive ; the majority produce $\alpha\beta$ haemolysins, are pathogenic to mice and produce fawn or golden pigment.

Of the bacteriophage types, X2, 88, S7 and UTB are common to both groups. These four types include 86 per cent. of the strains isolated from ewes and lambs, and 88 per cent. of the strains from cases of tick pyaemia.

TABLE VIII

THE BACTERIOLOGICAL EXAMINATION OF STAPHYLOCOCCI FROM NORMAL EWES AND LAMBS AND FROM TICK PYAEMIA CASES

| *Source of strains* | *Number of strains examined* | *Coagulase* | *Haemolysis* | *Pathogenicity* | *Pigment* | *Bacteriophage type* |
|---|---|---|---|---|---|---|
| Swabs from ewes and lambs | 211 | + 211 | αβ 209 | + 203 | Fawn 178 | X2 100<br>88 23<br>UTA 13<br>UTB 4<br>S7 4<br>UT 5<br>M 29 |
| | | | | | Golden 24 | X2 4<br>UTA 5<br>UTB 3<br>S7 1<br>M 11 |
| | | | | | White 1 | S7 1 |
| | | | | — 6 | Fawn 5 | X2 1<br>88 1<br>S7 3 |
| | | | | | White 1 | X2 1 |
| | | | α 1 | — 1 | Fawn 1 | M 1 |
| | | | β 1 | + 1 | Fawn 1 | X2 1 |
| Tick pyaemia cases | 56 | + 56 | αβ 53 | + 53 | Fawn 43 | X2 17<br>88 12<br>UTB 1<br>S7 1<br>UT 4<br>M 8 |
| | | | | | Golden 10 | 88 1<br>S7 3<br>42C 1<br>M 5 |
| | | | α 2 | + 2 | Fawn 2 | X2 1<br>88 1 |
| | | | β 1 | + 1 | Fawn 1 | M 1 |

*Abbreviations*: UT = untypeable. M = not submitted to phage typing.

The close correlation between the bacteriological properties of the haemolytic staphylococci isolated from external infections in ewes and lambs and those from cases of tick pyaemia, is strong evidence that they are the same species. This observation is confirmed by the fact that in 88 per cent. of the strains from pyaemia cases the bacteriophage types are the same as those in strains from ewes and lambs.

## DISCUSSION

The foregoing experiments show that the tick is unlikely to act as a true vector of the causal staphylococcus in tick pyaemia, and that the probable source of the organism is a skin infection on the lambs.

As staphylococcal pyaemia of this nature is unknown in tick-free districts it would appear that tick-bite is the decisive factor in the introduction or generalisation of the staphylococcal infection. There appear to be three possible methods by which this generalisation occurs : (*a*) the contaminated mouthparts of the tick may penetrate a blood vessel and set up a blood-borne infection directly ; (*b*) the salivary secretion of the tick may contain a factor which lowers the resistance to invasion of the blood stream by staphylococci from a local skin lesion ; (*c*) the breakdown in the local barrier to staphylococcal infection may be brought about by the lowering of the lambs' resistance caused by other tick-borne disease such as louping-ill and tick-borne fever.

In future experiments it is hoped to obtain more information on the relationship of tick-infestation to the development of this disease.

## SUMMARY

Experiments with infected ticks showed that the tick is unlikely to act as a true vector of the casual staphylococcus in tick pyaemia. In ticks infected by feeding on guinea-pigs suffering from a staphylococcal septicaemia, the infection did not survive the moulting period.

Haemolytic staphylococci were found to be of common occurrence in the natural orifices and skins of ewes and on the skins of lambs.

The staphylococci from the above sites were shown to have the same characteristics as the staphylococci from tick pyaemia cases.

## ACKNOWLEDGMENTS

I wish to thank Dr. J. Russell Greig, C.B.E., for his continued interest and encouragement.

I am also indebted to Mr. H. Williams Smith, of the London School of Hygiene and Tropical Medicine who tested many of the strains of staphylococci for bacteriophage type, and to Mr. Nigel Macmillan, The

Knowe, Kirkconnel, Dumfriesshire, who made his sheep stock available for experiment.

REFERENCES

Bryce, L. M., and Rowntree, P. M. (1936). *J. Path. Bact.*, **43**, 173.
McDiarmid, A. (1946). *Vet Rec.*, **58**, 103, 243 and 431.
M'Fadyean, J. (1894). *J. comp. Path.*, **7**, 207.
Williams, R., Clayton-Cooper, B., Duncan, J. McK., and Miles, E. M. (1943). *Lancet*, **1**, 522.
Williams, R., Clayton-Cooper, B., Faulkner, H. C., and Thomas, H. E. (1944). *Ibid.*, **1**, 787.

[*Received for publication March 29th*, 1947]

# AN IMMUNOLOGICAL STUDY OF *PASTEURELLA SEPTICA*

By

R. S. Roberts,

*The Evans Biological Institute, Runcorn, Cheshire*

## INTRODUCTION

This work was undertaken in an endeavour to ascertain whether adverse reports regarding the value of *Past. septica* immunisation might be due to the existence of antigenic types within the species.

A classification of *P. septica* based on fermentation reactions was suggested by Jones (1921) but was not supported by the work of Edington (1930). Spray (1923), Mørch and Krogh-Lund (1930), Newsom and Cross (1932) and Khalifa (1935) found strain differences by cultural and biochemical methods, but Gochenour (1924), Tanaka (1926), Frohböse (1926) and Bringham and Rettger (1935) found no reason to differentiate types on these grounds. Differences were detected by agglutination and agglutinin absorption by Cornelius (1929) and Battelli (1944), and by the precipitin test by Yusef (1935). The complement fixation test gave some success in the hands of Matsuda (1910), Roderick (1922), Tanaka (1926) and Lal (1927). Pribram and St. Plassaj (1921) suggested a classification based on motility. Some evidence in the literature indicates that differences between strains may be detected by the absence of cross protection (Wassermann and Ostertag, 1902; Schirop, 1908; Meissner and Shern, 1910; Battelli, 1944). Evidence that there is cross protection even between strains from different hosts is also abundant (Klepzow, 1903–04; Chamberland and Jouan, 1906; Citron and Putz, 1907; Gallagher, 1917; Gochenour, 1924). No serious attempt appears to have been made to study the species from an immunological angle, and although Lignières (1906) suggested a classification based on immunity, this is seldom suggested by other workers.

The difficulties inherent in the estimation of immunity to non-toxinogenic organisms probably accounts for the fact that this phenomenon is seldom used in differentiating types in any bacterial species. The writer's experience in the titration of anti-bacterial sera seemed to indicate that, provided immunity was sufficiently great to allow the use of large infecting doses, satisfactory results could be obtained by protecting animals with graded doses of serum and infecting them later with a massive dose of culture. The work recorded here is based on this principle of studying the anti-infective properties of immune sera.

## PLAN OF RESEARCH

Twenty virulent strains of *P. septica* were obtained from several countries and anti-serum was prepared with each strain. (Table I.) A preliminary search for gross immunological differences was made by protecting groups of four or five mice with large doses of each of the first ten antisera prepared and infecting a group with each of the strains in the series. This preliminary selection indicated

that some strains were closely related, whereas others showed such marked immunological differences that it would have been useless to attempt to demonstrate cross protection between them. It was also possible to detect similarities in the protection afforded by a given serum against groups of strains. A close examination of these results was made from time to time and suitable pairs were selected for comparison by cross protection tests. When the strains had been segregated by this means, a more detailed study was undertaken to ascertain the extent of the immunological differences between the groups and finally to determine whether the differences were partial or absolute. In this way, the 20 strains were separated into four groups which were designated Types I, II, III and IV respectively. (Table I.) Antisera prepared with Type I strains protected

TABLE I

SUMMARY OF STRAINS INVESTIGATED WITH STRAIN-SPECIFIC ANTISERA

| *Strain No.* | *Type* | *Host* | *Country of origin* | *Usual m.l.d. in ml.* |
|---|---|---|---|---|
| 214 | I | Bovine | India (1941) | $10^{-8}$ |
| 215 | „ | Bovine | India (1942) | $10^{-8}$ |
| 216 | „ | Bovine | India (1942) | $10^{-6}$ |
| 231 | „ | Porcine | Spain | $10^{-8}$ |
| 232 | „ | Porcine | Spain | $10^{-8}$ |
| A4 | II | Avian | Great Britain | $10^{-8}$ |
| Avis III | „ | Avian | Italy | $10^{-8}$ |
| 310 | „ | Bovine | U.S.A. | $10^{-5}$ |
| 209 | „ | Avian | France | $10^{-8}$ |
| 212 | „ | Avian | Holland | $10^{-8}$ |
| 222 | „ | Avian | Denmark | $10^{-9}$ |
| 224 | „ | Avian | Spain | $10^{-8}$ |
| 225 | „ | Avian | Spain | $10^{-10}$ |
| 226 | „ | Bovine | Spain | $10^{-8}$ |
| 227 | „ | Porcine | Spain | $10^{-8}$ |
| 229 | „ | Avian | Spain | $10^{-8}$ |
| 230 | „ | Avian | Spain | $10^{-8}$ |
| P1 | III | Bovine | Great Britain | $10^{-7}$ |
| 211 | „ | Deer | U.S.A. | $10^{-8}$ |
| 739 | IV | Bovine | Great Britain | $10^{-8}$ |
| 218* | — | Avian | India | $10^{-6}$ |

* This strain was added to the series in the course of the investigation.

mice against this type only, and the antisera prepared with strains of other types did not protect against infection with Type I strains. Types II, III and IV antisera conferred full protection against infection with all the strains in their own type, and a slight cross protection could be demonstrated between the three types.

These results were used as a basis for the typing of 14 further strains and for the examination of three samples of desiccated anti-

serum preserved from an investigation which had been interrupted seven years earlier.

Cultural and biochemical studies were carried out concurrently.

## EXPERIMENTAL METHODS

*Characters of the Strains.*—The 34 strains used for immunological studies, and 12 Eritrean strains (Battelli, 1944) were found to conform to the classical description of the species in their morphology, motility, capsulation and growth on broth, plain agar, blood agar and in stab culture in gelatin, in their failure to grow on solid McConkey medium, production of indole in broth culture, negative methyl red reaction, fermentation of glucose, saccharose, mannite and laevulose and failure to ferment lactose and salicin.

TABLE II

AN EXAMPLE TAKEN FROM LABORATORY RECORDS OF A CROSS PROTECTION EXPERIMENT BETWEEN STRAINS OF ONE TYPE (*Strains* 214 *and* 232)

| *Antiserum* | *Culture* | *Result* |
|---|---|---|
| 0·01 ml. of 214 | 0·01 ml. of 214 | — — — — — — — — — — — |
| 0·002 „ | „ | + 24 hours + + 48 hours + + + 72 hours — — — — |
| 0·004 „ | „ | + + + + + + + + + + + 24 hours |
| 0·01 ml. of 214 | 0·01 ml. of 232 | — — — — — — — — — — — |
| 0·02 „ | „ | + + + + 24 hours + + 48 hours + 72 hours — — — |
| 0·004 „ | „ | + + + + + + + + + + + 24 hours |
| 0·5 ml. of 232 | 0·01 ml. of 214 | — — — — — — — — — — — |
| 0·1 „ | „ | — — — — — — — — — — — |
| 0·02 „ | „ | + + + 24 hours + + + 48 hours + + + 72 hours — |
| 0·5 ml. of 232 | 0·01 ml. of 232 | — — — — — — — — — — — |
| 0·1 „ | „ | + 24 hours — — — — — — — — — — |
| 0·02 „ | „ | + + + 24 hours + + + 48 hours + + + 72 hours — |

| | *Infectivity of cultures* | |
|---|---|---|
| | 214 | 232 |
| $10^{-6}$ ml. | + + + + 24 hours + 48 hours | + + + + + 24 hours |
| $10^{-7}$ ml. | + + + 24 hours + + 48 hours | + + + 48 hours — — |

+ = Died. — = Lived

Three strains were bipolar elongated rods with pointed ends in contrast to the normal coccoid form. In three strains indole could be detected only by resorting to the technique suggested by Spray (1923). The appearance of a small mound in the centre of the colony surface on the second or third day of incubation on surface media, was found to be of assistance in identification. Delayed fermentation appeared to be common in saccharose and sorbitol. The character of broth growth and the fermentation reactions in arabinose, xylose, maltose, trehalose, dulcitol and sorbitol, corresponded in variable degrees with immunological differences and are discussed later.

*Maintenance and propagation of strains.*—All strains were kept in the dried state and in 10 per cent. blood broth at – 4° C. Broth cultures were

made from the dried cultures every two or three weeks and these were available for day-to-day use. The broth used throughout this work was a papain digest of horse muscle standardised to a total solids content of 2 per cent., with 0·2 per cent. of glucose and 0·5 per cent. of NaCl added. Horse-blood agar with the same nutrient basis was employed in check tests for purity and in the detection of small numbers of viable organisms (Webster, 1925).

*Preparation of antisera.*—Antisera against six strains were prepared in horses and against the remaining 14 strains in rabbits. Broth cultures grown at 38° C. and killed by heating at 56° C. for one hour (Schütze, 1932) were injected intravenously twice weekly. In most cases eight injections were given, increasing from 0·5 ml. to 5 ml. for rabbits and from 2 ml. to 60 ml. for horses. A second course of injections was sometimes necessary. The following are the approximate quantities of serum which usually protected 50 per cent. of mice against an infecting dose of about one million m.l.d.s of the various strains: 1·0 ml. (one), 0·5 ml. (one), 0·2 ml. (one), 0·1 ml. (four), 0·04 ml. (six), 0·02 ml. (six), 0·005 ml. (one). Serum was withdrawn seven days after the final injection. Preservative was added and the protective value of the serum against the homologous strain determined.

*Method of infecting mice.*—The standard method used in all cross protection tests and potency assays was the intraperitoneal injection of 0·01 ml. of six-hour-old or 18-hour-old broth culture. In all such experiments it was confirmed that the strain was at its customary level of virulence by the injection of groups of two, four or five mice with two or three serial dilutions of culture. This infecting dose, when prepared from six-hour culture, contained from 100,000 to100,000,000 m.l.d.s.* The error in the preparation of each $\times$ 10 dilution was maintained at $\pm$ 1 per cent. The 18-hour cultures were ten to 100 times less virulent, and were used mainly in exploratory experiments. Strains 310, P1 and 216 were generally ten to 100 times less virulent than the remainder (Table I). Viable organisms, demonstrated by plating on blood agar, were almost invariably present in one part in one million of the infecting dose of culture. It appears probable that the majority of the strains used were not lethal for mice as single organisms. This view is based on the fact that broth cultures of one or two strains sometimes killed all mice in a dose of $10^{-10}$ ml.; similar cultures of the majority of strains killed only a proportion of mice at $10^{-9}$ ml., and it seems probable therefore that this dose contained at least ten organisms.

It was found that the capacity of an organism to overcome the protection afforded by anti-serum was not necessarily proportional to its virulence and some strains of consistently high virulence appeared to have only a relatively low power of overcoming serum protection. This property is referred to as "aggressiveness."

Unprotected mice injected with the full infecting dose died frequently within six hours and mice infected with ten to 100 m.l.d.s generally died in less than 24 hours. There was some strain variation in survival times and mice infected with one strain (P1) survived 24 hours longer than those

* The expression "m.l.d." is used solely as a form of speech to indicate amounts of culture which caused death in a substantial proportion of mice. Theoretically, it is doubtful whether organisms should be present in $10^{-11}$ ml. of broth culture, and some strains killed five out of five mice in a dose of $10^{-10}$ ml. An estimation of the "a.l.d." of such a culture therefore could only reflect the chance presence or absence of viable organisms in the lower dilutions.

infected with a similar number of m.l.d.s of other strains. The result of the injection of culture into mice could be appreciated with moderate accuracy 24 hours after injection and few changes occurred after 48 hours.

*Type identification by mouse protection tests.*—Mice were injected subcutaneously with serial quantities of each serum and 24 hours later were infected by intraperitoneal injection of 0·01 ml. of a six-hour-old broth culture, half of each series with the homologous and half with the heterologous culture. The infected mice were observed for three days.

Previous work had indicated that to obtain clear differences in protection the serial quantities of serum injected into groups of mice should increase by multiples of four or five, and this was confirmed by the following experiment :—

| Serum dilution ... ... ... | Undil. | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 |
|---|---|---|---|---|---|---|---|
| Percentage survival in groups of 20 mice infected with culture | 95 | 100 | 95 | 95 | 35 | 0 | 0 |

It is seen that when the doses of serum are increased in multiples of two, the result may not be critical unless a very large number of mice is used. Increases of $\times 4$ on the other hand, give a clear difference of (1) survival with the larger dose and partial survival with the smaller dose ; (2) total death at one level and partial survival at the other, or (3) almost complete protection at the higher dose and a complete mortality at the lower dose if the doses are fortunately selected as in this experiment. Differences of $\times 5$ were used in all cross protection tests and potency assays. In some cross-protection experiments the number of mouse groups protected with each serum could be limited to four, but it was generally preferred to employ six groups in order to cover unexpected variations in the infectivity of the culture, *i.e.*, 120 mice were used as principals in each cross-protection test. It was not necessary to use identical quantities of antiserum against each of the two strains, but they were arranged to facilitate a quantitative comparison of the results. Experiments carried out in this pattern were not difficult to interpret and it was generally found that the antisera of two strains, which had been suspected as being of one type in the preliminary selection, protected in the same proportions against the two infecting cultures. Thus, if A antiserum protected against A at x ml. and against B at 5 x ml., it was found that B antiserum protected against A at y ml. and against B at 5y ml. The result of such an experiment is reproduced in Table II, which gives an indication of the corroborative evidence which is obtained from observations of survival times. Thus, at three points, only one mouse survived, but it will be seen that the significance of the result is confirmed by the delayed death of other mice in the group. Similarly, the death of one or two mice in a group in which the remaining mice survive, may be rendered less significant by the fact that the deaths occur early as in the last group but one in this table ; 98 per cent. of mice in such tests were either dead or in normal health on the third day. At the termination of an experiment sick mice were recorded in laboratory notes in two degrees, but to simplify recording, these have been entered in the tables as living or dead according to the degree of illness recorded.

This test could be adapted to detect smaller degrees of protection by reducing the amount of culture used to infect the mice. In order to appreciate the significance of such a reduction it is necessary to have an approximate knowledge of the effect upon the protective value of the antiserum. The experiment recorded in Table III is a comparison of the effects of an anti-serum against $10^{-2}$ ml. and $10^{-6}$ ml. of culture, and indicates that

within the limits in which the infecting strain is highly pathogenic, the amount of antiserum required to give the same degree of protection is reduced by 50 times when the infecting dose is decreased 10,000 times.

When it became apparent that two strains or two types were unrelated, final evidence of absence of cross protection was obtained by treating groups of ten mice with a fixed dose of antiserum and infecting one group with a serial dilution of the heterologous culture. Such a test is illustrated in the experiment in Table IV.

TABLE III

THE RATIO BETWEEN THE AMOUNT OF CULTURE AND AMOUNT OF ANTISERUM

| *Antiserum* | *Percentage survival in groups of ten mice infected with* 10,000,000 *m.l.d.s* | *infected with* 1,000 *m.l.d.s* |
|---|---|---|
| 0·01 ml. | 100 | — |
| 0·002 „ | 40 | — |
| 0·001 „ | — | 100 |
| 0·004 „ | 0 | — |
| 0·0002 „ | — | 90 |
| 0·00004 „ | — | 40 |

— indicates that no test was carried out at that level.

## RESULTS

The final classification of the 20 strains by these methods showed Type I, five ; Type II, twelve ; Type III, two ; and Type IV, one (Table I).

### *Type I*

Cross protection experiments in the form illustrated in Table II demonstrated that 214 and 215, 214 and 232, 232 and 231, and 214 and 216 were immunologically identical pairs ; by relating all the strains to the type strain 214, all the strains were concluded to be identical. The results of one of these tests is shown in Table V.

During exploratory experiments the following facts were established by experiments on groups of four or five mice.

(1) Every Type I antiserum conferred protection against the infecting dose of all Type I strains, in doses not far different from those required for homologous protection.

(2) The Type I antisera, in doses one hundred to one thousand times greater than those required for homologous protection failed to confer protection against the infecting dose of any of the remaining 15 strains in the series.

(3) The antisera of six Type II strains, the two Type III strains and the Type IV strain conferred no protection against the infecting dose of any Type I strain.

An experiment was carried out to demonstrate the failure of

Type I serum to protect against Type II strains. Groups of ten mice were protected with 0·01 ml. of the Type I serum 214/215. This amount is probably one thousand times greater than would be required to protect against 10 and 100 m.l.d.s of a Type I culture (see Table III), and in control groups in this experiment this amount did, in fact, give 100 per cent. protection twice in groups of ten mice against one to ten million m.l.d.s of a Type I culture. Groups of protected mice and similar groups of control untreated mice were infected with each Type II culture using amounts which were expected to lie close to the infective limit of each strain, *e.g.*, 1, 10 and 100 m.l.d.s. The serum did not afford protection against any degree of infection with any one of the Type II strains. The mortality in 360 treated mice was 74·2 per cent. and in 360 control mice, 72·6 per cent.

TABLE IV

AN EXAMPLE OF THE EXPERIMENTAL PROCEDURE EMPLOYED TO DETERMINE ABSENCE OF CROSS PROTECTION
(Types I and IV)

| *Antiserum* | *Culture* | *Number of mice* | *Percentage survival* |
|---|---|---|---|
| 0·01 ml. of 739 | $10^{-3}$ ml. of 214 | 10 | 0 |
| „ | $10^{-5}$ „ | 10 | 0 |
| „ | $10^{-7}$ „ | 10 | 0 |
| „ | $10^{-9}$ „ | 10 | 100 |
| None | $10^{-7}$ „ | 4 | 0 |
| None | $10^{-9}$ „ | 4 | 75 |
| 0·01 ml. of 739 | $10^{-2}$ ml. of 739 | 10 | 100 |
| 0·01 ml. of 214 | $10^{-3}$ ml. of 739 | 10 | 0 |
| „ | $10^{-5}$ „ | 10 | 10 |
| „ | $10^{-7}$ „ | 10 | 20 |
| „ | $10^{-9}$ „ | 10 | 100 |
| None | $10^{-7}$ „ | 4 | 25 |
| None | $10^{-9}$ „ | 4 | 100 |
| 0·01 ml. of 214 | $10^{-2}$ ml. of 214 | 10 | 100 |

An experiment was carried out to ascertain whether Type II sera had any protective action against a small dose of a Type I culture. A group of ten mice was injected with an amount of each Type II serum which would be expected to protect against an injection of ten million m.l.d.s of normally aggressive Type II cultures. For most strains a further group was protected with larger amounts. Each group was infected with about 100 m.l.d.s of culture of strain 214. There was evidence of partial protection with some sera, but it was noticed that this had occurred only with the sera produced in horses.

It had not been anticipated at first that serum protection experiments would be carried out with small infecting doses of culture and the only evidence available concerning the serum producing animals

was that 1 c.c. of their serum before immunisation had no effect upon the course of the infection initiated by 0·01 ml. of culture. When it was found that the Type II sera which had been produced in horses (A4, Avis III, 310 and 212) conferred some immunity against a Type I strain, it became necessary to ascertain whether normal serum also could confer this degree of immunity. Samples of serum were obtained from 45 horses from widely separated areas of the British Isles. Duplicate groups of four or five mice were injected with 0·2 c.c. of each sample and one group for each serum was injected with 100 to 1,000 m.l.d.s of A4 culture (Type II) and 214 culture (Type I) respectively. From a total of 200 mice so treated and infected with A4 culture, only five mice were alive at 24 hours and none survived to the 48th hour. Of the 200 mice which received the same sera, but which were infected with the 214 culture, 27 mice, or 13·5 per cent., survived and 18, or 9 per cent. of the remainder, lived longer than 48 hours ; one serum protected five mice out of five, and in a further 17 sera, evidence of protection could be deduced from the survival rate and the survival times. Similar tests carried out on 20 samples of normal rabbit serum gave no evidence of protection against either strain. Thus, the fact that 40 per cent. of normal horse sera conferred some protection against Type I infection, appears to explain the immunity conferred by samples of Type II sera prepared in horses against Type I infection.

The question of whether any cross protection occurred between Types I and II was considered to be of great importance and another experiment was carried out. By infecting groups of ten mice protected with the A4 horse antiserum, with serial quantities of 214 culture from $10^{-2}$ ml. to $10^{-9}$ ml., it was confirmed that this antiserum gave partial protection against 214 infection. Two new samples of A4 antiserum were prepared in rabbits. These sera were both shown to protect mice against ten million m.l.d.s of A4 culture in a dose of 0·04 ml. Mice protected with 0·2 ml. of these sera had no resistance to minimal infection with strain 214. There is thus no evidence of any immunological relation between Type I and strains of Types II, III and IV (Table IV). Thus the immunological status of Type I strains is that they are distinct from all other strains in the series.

*Type II*

It was demonstrated that the 12 strains of this type were identical by carrying out cross protection tests in the manner illustrated in Table II. The strains were paired for these tests as follows : A4 and Avis III, A4 and 222, 212 and 222, A4 and 226, 226 and 224, A4 and 225, A4 and 227, 229 and 230, A4 and 229, and A4 and 310. It will be seen that in this way all the strains were finally compared with A4. Two examples of these tests are shown in Table V. Strain 310 appeared to differ slightly from the other strains and it is probable that it is not fully identical with other members of the type.

It is mainly among the Type II strains that variations in "aggressiveness" have been observed. In Table V an example is shown in the cross protection test between A4 and 209 in which infection with the A4 culture was more easily prevented. This could not be anticipated either from the commonly observed virulence of the two strains or from the behaviour of unprotected mice in the experiment.

TABLE V

EXAMPLES OF CROSS PROTECTION TESTS BETWEEN STRAINS OF THE SAME TYPE

| *Type* | *Antiserum* | | *Percentage survival in groups of ten mice infected with* $10^6$ *to* $10^8$ *m.l.d.s* | |
|---|---|---|---|---|
| | *Strain* | *Amount* | *Homologous culture* | *Heterologous culture* |
| I* | 214 | 0·2 ml. | 100 | 100 |
| | | 0·04 „ | 80 | 85 |
| | | 0·008 „ | 45 | 45 |
| | 215 | 0·2 ml. | 100 | 100 |
| | | 0·04 „ | 100 | 90 |
| | | 0·008 „ | 40 | 65 |
| II | A4 | 0·5 ml. | — | 70 |
| | | 0·1 „ | 80 | 0 |
| | | 0·02 „ | 0 | — |
| | 209 | 0·2 ml. | 100 | — |
| | | 0·04 „ | 70 | 100 |
| | | 0·008 „ | — | 70 |
| II | A4 | 0·2 ml. | 90 | 90 |
| | | 0·04 „ | 30 | 20 |
| | | 0·008 „ | 0 | 0 |
| | 222 | 0·2 ml. | 100 | 100 |
| | | 0·04 „ | 100 | 80 |
| | | 0·008 „ | 20 | 30 |
| III | 211 | 0·2 ml. | 60 | 100 |
| | | 0·04 „ | 0 | 70 |
| | P1 | 0·5 ml. | — | 60 |
| | | 0·1 „ | 90 | 0 |
| | | 0·02 „ | 60 | — |

— indicates no test at that level.

* Combined results of 2 replicates

A number of Type II antisera (A4, Avis 111, 310, 212, 225 and 227) were shown to confer protection against all Type II strains and the amounts required for protection were commensurate with the potency of the antiserum. Each failed to protect mice against infection with all the Type I strains. They protected mice against the Type III strain P1, but seldom against the more aggressive strain 211. Mice protected with 1 ml. of Type III serum (211), which

protected against the homologous strain in a dose of 0·02 ml., resisted infection with a few Type II strains but similar groups protected with 0·2 ml. of this serum and infected with each Type II strain were not protected. The P1 antiserum did not confer protection against Type II strains; but this was to be expected, as its potency was considerably less than that of the 211 serum. The Type IV serum, which gave protection against the homologous strain in a dose of 0·008 ml. seldom protected against Type II strains in a dose of 0·04 ml., and frequently failed to give protection in a dose of 0·25 ml. Some of the more potent Type II sera gave some protection against infection with the type IV strain.

The exact relationship between Type II and Type III and IV is illustrated in Table VI.

Thus, Type II strains are immunologically specific in relation to Type I. They show partial cross protection with Types III and IV.

*Type III*

Strains 211 and P1* have been placed in this type. Strain 211 was considerably more aggressive than P1 and was therefore used for most of the experimental work on this group. It is seen in Table V that there is cross protection between the two strains. It is probable that the relation is less complete than that between the members of the other types.

An experiment of the pattern of that shown in Table IV demonstrated that no cross protection occurred between 211 and the Type I strain 214. No Type I antiserum protected against infection with 211. Neither of the Type III antisera conferred any protection against any Type I strain.

The immunological relation between 211 and the Type II strain A4, shown in Table VI, is in no way comparable to the relation between two strains of the same type. The antiserum (211) gave some protection against all but the more aggressive of the Type II strains, and large doses of the more potent Type II sera protected against infection with strain 211.

Type III strains bear some immunological relation to the Type IV strain. Although Table VI appears to indicate that the cross protection between 211 and 739 was in one direction only this may have been due to a difference in aggressiveness. The antiserum of 739 also gave protection against P1.

Thus, while the two strains comprising Type III are immunologically distinguishable from all the other strains of the series, they have both shown some relation to Type II and Type IV strains.

*Type IV*

Strain 739* was the only one of this type in the series. A type

* This strain is described by Kyaw (1942).

number was assigned to it because the existence of two similar strains was presumed from evidence in later work.

This strain was unrelated to Type I (Table IV). It was distinguishable without difficulty from Type II and Type III strains, but was related to them (Table VI). The antiserum of 739 afforded protection against the less aggressive Type II strains in amounts

TABLE VI

EXAMPLES OF PARTIAL CROSS PROTECTION BETWEEN STRAINS OF DISSIMILAR TYPES

| *Type* | *Antiserum* | | *Percentage survival in groups of ten mice infected with* $10^6$ *m.l.d.s* | |
|---|---|---|---|---|
| | *Strain* | *Amount* | *Homologous culture* | *Heterologous culture* |
| II | A4 | 1·0 ml. | — | 0 |
| | | 0·2 „ | 70 | 0 |
| | | 0·04 „ | 20 | — |
| IV | 739 | 0·25 „ | — | 10 |
| | | 0·05 „ | 90 | 0 |
| | | 0·01 „ | 30 | — |
| III | 211 | 1·0 ml. | — | 0 |
| | | 0·1 „ | 80 | — |
| | | 0·02 „ | 10 | — |
| IV | 739 | 1·0 ml. | — | 20 |
| | | 0·2 „ | — | 10 |
| | | 0·05 „ | 90 | — |
| | | 0·01 „ | 30 | — |
| II | A4 | 1·0 ml. | — | 0 |
| | | 0·2 „ | 70 | 0 |
| | | 1·04 „ | 20 | — |
| III | 211 | 1·0 ml. | — | 80 |
| | | 0·2 „ | — | 0 |
| | | 0·1 „ | 80 | — |
| | | 0·02 „ | 10 | — |

— indicates that no test was carried out at that level.

varying from five to 25 times that required for homologous protection, but it failed to protect against the more aggressive strains in an amount 125 times greater than that required for homologous protection. Similarly, large doses of some Type II sera gave some protection against infection with 739.

Thus, 739 is clearly distinguishable from Type II and Type III strains, but is related to them. It has no immunological relation to Type I strains.

* This strain is derived from the outbreak of pasteurellosis in calves described by Bythell (1945).

### THE TYPING OF UNKNOWN STRAINS *

An attempt was made to evolve a method of examining new strains with a view to ascertaining whether they could be assigned to the types described, without preparing antiserum from them. From the experience gained it is concluded that there is no available method of establishing the immunological status of a strain of *P. septica* without preparing a specific antiserum and carrying out a cross protection test on the scale employed in this work, but it is believed that a method of sufficient accuracy for clinical purposes can be evolved. This consists in inoculating the unknown strain into mice protected with known antisera. The new strain, in a dose of 0·01 ml. of a six-hour culture, is used to infect mice given an amount of each type serum which is twice as great as that which protects against a highly pathogenic homologous strain. At the same time, an approximate value is obtained for the virulence of the new strain by infecting groups of two normal mice with $10^{-3}$, $10^{-5}$, $10^{-7}$, and $10^{-9}$ ml. of culture respectively. If the mice infected with $10^{-3}$ ml. of culture do not die the experiment may be repeated using an infecting dose of 0·1 ml. or more. If, on the other hand, the virulence of the strain is within the range tested and a group of treated mice survives, a further test may be carried out to obtain an approximate value for the protective power of the type serum for the new strain and to ascertain whether this bears any relation to the known anti-infective titre of the serum. If a strain requires for protection an amount of type antiserum which is abnormally high for a strain of that grade of pathogenicity, it must give rise to doubt as to whether the strain belongs to that type. Fourteen strains of *P. septica* were typed by this method. Four were assigned to Type I, four to Type II and one was classified as " related to " Type IV. None of the type sera gave protection against the remaining five strains and it was concluded that they were new varieties. Antiserum was prepared against one of these (218), and it was confirmed that it was unrelated to the types described. The other unidentified strains included the only two ovine strains, the only strain from a rabbit, and one avian strain of Australian origin.

### IDENTIFICATION OF THREE SAMPLES OF DESICCATED ANTISERA.

This investigation had been commenced in 1939. When it was interrupted the cultures were lost, but three samples of antiserum were desiccated and stored.

These antisera were examined to discover their protective properties against a strain of each of the four types, and the results obtained were compared with those obtained when the same antisera were studied in conjunction with their own cultures in 1939. The results, summarised in Table VII, indicate that the Italian bovine

* These experiments were carried out before any cultural or biochemical differences had been revealed.

strain C.R. was a Type I strain, the British strain " aviseptica " was a Type II strain and that the British bovine strain " Wortley "* was a Type IV strain. The absence of protection by the " C.R." serum against Type II, Type III and Type IV infections, and by the " Wortley " and " aviseptica " sera against Type I infection was apparently absolute. The degree of protection conferred by the " Wortley " serum against Type IV infection, by the " C.R." serum against Type I infection and by the " aviseptica " serum against Type II infection was comparable in degree to the homologous protection observed with the same sera in 1939. It is probable, therefore, that the results of the present tests are a true indication of the identity of the strains investigated in 1939.

TABLE VII

IDENTIFICATION OF DESICCATED ANTISERA

| *Antiserum* | *Strains used for infection* | | | | | | | *Conclusion* |
|---|---|---|---|---|---|---|---|---|
| | *In* 1939 | | *In present investigation* | | | | | |
| | *Wortley* | *"C.R."* | *Avi-septica* | *Type I* | *Type II* | *Type III* | *Type IV* | |
| Wortley | + | — | — | — | — | — | + | Type IV |
| " C.R." | — | + | — | + | — | — | — | Type I |
| Aviseptica | — | — | + | — | + | — | — | Type II |

+ = Protection
— = No protection

## AN EXAMINATION OF FACTORS CORRELATED WITH THE IMMUNOLOGICAL GROUPING

As shown in Tables I and VIII, an immunological classification of the 34 *P. septica* strains examined indicates a loose relationship with the zoological classification of the hosts. Strains of avian origin were mainly of Type II ; bovine and porcine strains were generally of Type I. Two avian strains, from India and Australia, each appeared to represent a new type. Two ovine strains and one strain from a rabbit also failed to conform with existing types.

Considerable importance would be attached to any correlation between the immunological classification and the cultural and biochemical characters of the strains. Because of this, earlier work on the latter characters of the grouped strains was examined and repeated. The nature of growth in broth culture appeared to offer promise of a correlation. Growth of *P. septica* in broth is usually described as evenly turbid at first with sedimentation in a few days. It had been observed that the growth varied considerably from strain to strain and that while some strains remained evenly turbid for

* This strain was described by Lovell (1939).

several weeks, others sedimented completely in a few days. A further study of this character, carried out on 29 strains, showed that although the correlation was incomplete the majority of strains, which maintained even turbidity for one week or more, were Type II. If by further work the conditions under which this differentiation occurs could be defined, it is possible that it might prove of assistance in the determination of types. It does not appear to be a " rough " and " smooth " phenomenon.

TABLE VIII

SUMMARY OF THE RELATION BETWEEN HOST AND TYPE IN 34 STRAINS AND THREE PRESERVED ANTISERA*

| | *Type I* | *Type II* | *Type III* | *Type IV* | *Other Types* | *Totals* |
|---|---|---|---|---|---|---|
| Bovine ... ... | 7 | 4 | 1 | 2 | 0 | 14 |
| Avian ... ... | 0 | 12 | 0 | 1 | 2 | 15 |
| Pig ... ... | 3 | 1 | 0 | 0 | 0 | 4 |
| Deer ... ... | 0 | 0 | 1 | 0 | 0 | 1 |
| Sheep ... ... | 0 | 0 | 0 | 0 | 2 | 2 |
| Rabbit ... ... | 0 | 0 | 0 | 0 | 1 | 1 |
| Totals ... ... | 10 | 17 | 2 | 3 | 5 | 37 |

* Since the conclusion of this work a British bovine strain has been typed as Type I, a British strain from a goose as Type II, and a British human strain of low virulence has been shown to be distinct from the described types.

Three stages of sedimentation were recognised, namely (1) a slight deposit ; (2) a heavy deposit ; (3) a clear supernatant. The " heavy deposit " stage appeared to be the most critical ; and the following is a summary of the times at which it was reached in the 29 cultures observed.

Type I 3, 4, 4, 4, 4, 4, 5, 5, and 40 days.
,, II 4, 8, 11, 13, 19, 20, 20, 29, 33, 40, 40, 40, 40, 40 days.
,, III 4, 4 days.
,, IV 3, 6 days.
Strains 218 and 262 : 3, 5 days.

Cultures which remained turbid for 30 days or more were of course affected by evaporation. Strain 310 (Type II) which was of the early sedimenting type, was also atypical in immunological tests, and was a dulcitol fermenter.

It appears probable that a correlation exists between the immunological grouping and some fermentation reactions. The 34 strains which have been investigated by immunological methods were uniform in their reactions in glucose, lactose, mannite, salicin, saccharose and laevulose. Among the total of 46 strains examined, the Australian avian strain 262 was the only one which fermented maltose. This strain was also one of four which fermented dulcitol. Protection against this strain could not be demonstrated with any type serum or with the antiserum of the Indian avian strain 218 :

four attempts to prepare an anti-serum with it failed. Out of 46 strains two only fermented trehalose; one of these was the avian 218 and the other was the unclassified strain from a rabbit. Seventeen strains* classified as Type II by immunological tests fermented arabinose whereas all the ten strains classified as Type I, and two Type III strains were negative; the main Type IV strain was positive but 242, believed to be immunologically related to it, was negative. Strains 218 and 262, and the other strains classified as "new types," were also negative. Type I and Type III strains all fermented xylose, but 14 out of 17 Type II strains did not ferment it. The type IV strain gave an indefinite result. We were not satisfied with the results obtained with xylose; delay in fermentation and the development of slight acidity in all tubes made the tests difficult to read for the first four days, and suggested that the reagent may have been impure. Four only out of 46 strains failed to ferment sorbitol, and these were all in Type II. Dulcitol was fermented by strains 739, 310 and the Australian strain 262; all other strains failed to ferment it.

It appears probable from these results that with the accumulation of data on a larger number of strains, it may be possible to recognise some of the immunological types by means of *in vitro* tests. The characters noted in this investigation are summarised below.

| *Strains* | *Characters* |
|---|---|
| Type I ... ... ... | Arabinose negative, xylose positive. |
| ,, II ... ... ... | Arabinose positive, xylose generally negative. Slow sedimentation in broth. |
| ,, III ... ... ... | As for Type I. |
| ,, IV ... ... ... | Arabinose positive, dulcitol positive, xylose doubtful. |
| Strain 218 ... ... ... | Arabinose negative, trehalose positive. |
| ,, 262 ... ... ... | Arabinose negative, dulcitol positive, maltose positive. |
| Rabbit strain 253 ... ... | Arabinose negative, trehalose positive. |
| Unidentified ovine strains | Arabinose negative. |

## DISCUSSION

The essential aim of this work was to establish the fact that immunological types existed within the species *P. septica*, and it is not intended that they should demonstrate the constitution of the species; in fact, it is clear from the examination of strains not included in the main series of 20 that immunological types exist which are not represented in this series. Nevertheless, it appears probable that Types I and II are of aetiological importance in widespread areas of the world. It is perhaps early to speculate on the probable constitution of the species, but there is some suggestion

* These included one strain from the Eritrean collection.

in the results that it may not be unlike that of the salmonella group, with its wide variation of types, dominated by a few commonly occurring members.

Some early authors, quoted by Schütze (1929), claimed that the immunity conferred by antiserum was not a true immunity and that protected animals died in one or two weeks. It has been observed in this work that in groups of mice which were partially protected by serum for three days, deaths from pasteurellosis might continue to occur sporadically for 14 days after infection, but that mice protected with quantities of antiserum which gave complete protection for three days, survived and continued to gain weight throughout an observation period of six weeks. It is possible that the delayed deaths may have been due to re-infection as passive immunity diminished.

Comprehensive reviews of the literature on haemorrhagic septicaemia and fowl cholera will be found in publications by Tanaka (1926), Lal (1927), Schütze (1929), Kyaw (1942), and Battelli (1944). It does not appear to be difficult to reconcile the reports on immunity with the results of the experimental work recorded here. Wasserman and Ostertag (1902) who reported failure to protect pigs with antiserum prepared from a porcine strain, may have been dealing with variations in type or in aggressiveness. Klepzow (1904) and Chamberland and Jouan (1906), in asserting that one strain conferred immunity against others, may have been dealing with strains of identical types. Schirop (1908), who advocated protection with homologous serum, may have had experience of strains of differing types or differing aggressiveness. The report by Gallagher (1917) that an avirulent avian strain conferred immunity against bovine and porcine strains is unexpected, but it can be reconciled by assuming that the avian strains like the majority of ours was of Type II, and that the porcine and bovine strains were also of this type. Strains from cattle and buffaloes have fallen generally into Type I, and there is little difficulty, therefore, in reconciling the findings of Gochenour (1924) that a strain from a buffalo was identical with bovine strains, or those of Jacotot (1940) that antiserum prepared from a bovine protected against other bovine strains and that field prophylaxis in cattle was successful. The contention of Hutyra (1920) that all strains should be regarded as identical may have been based on experience with one type ; this does not seem improbable, when it is realised that 12 out of 20 strains in our main series fall into one type and were obtained from a number of hosts.

The 46 strains examined were remarkably constant in their morphological, cultural and biochemical characters over a wide range of tests. There were, however, some differences between the strains which were closely correlated with the immunological classification, and it is probable that if the rules of nomenclature were strictly applied, it would be necessary to regard Type I and Type II strains as separate species. It would follow from this that some of the single strains which appear to represent new types and

which have shown distinctive fermentation reactions, might also attain the status of species. Hence, to regard these types as species would offer a prospect of a new and complicated system of nomenclature in a subject which is already over-burdened. It was decided therefore, that even if it was not entirely correct to do so, it was preferable to adhere to the name of *Pasteurella septica* and to distinguish the immunological types by numbers.

It is clear from the results of this work that a monovalent antigen or antiserum can have little value for general use in prophylaxis. Even in employing polyvalent antisera and antigens, it would be difficult to decide on a combination which could be expected to give satisfactory results in all outbreaks ; the inclusion of too many types would reduce the amount of each ingredient, while the omission of types would undoubtedly lead to failure in some outbreaks. The observations made in this limited survey are not sufficiently complete to warrant any final recommendation for prophylaxis, but it is suggested that until more information is available, a Type II antigen might be employed for prophylaxis in fowls and a combined antigen, prepared from Type I and II strains, might be used for cattle and pigs. It is, of course, possible that some types may be found to have a regional distribution and in this case prophylaxis based on a knowledge of the local types would be expected to give satisfactory results.

## SUMMARY

Thirty-seven strains of *P. septica* were studied by cross-protection experiments in mice. By using 21 strain-specific antisera it was possible to classify them according to immunological differences. Although the majority was found to belong to two main types, it is probable that seven or eight types were represented in the collection. The classification had a loose relation to the species of the host and it was supported by a number of cultural and biochemical differences.

Ten Type I strains of bovine and porcine origin were type specific. They failed to ferment arabinose, and immune bodies corresponding to them were present in normal horse serum.

Seventeen Type II strains were mainly of avian origin, but included bovine and porcine strains. Partial cross protection occurred between this type and Types III and IV. Fermentation of arabinose, maintenance of turbidity in broth culture and absence of protective action by normal horse serum were characters of the group.

Two Type III strains from a bovine and a deer respectively, were related immunologically to Types II and IV.

Two Type IV strains were of British bovine origin and a further British strain from a turkey was related to them ; the type strain fermented dulcite.

An Indian avian strain was the only representative of its type ; this and a rabbit strain were the only trehalose fermenters.

An Australian avian strain was unrelated to any of the above ; it was the only maltose fermenter and it fermented dulcite.

The two remaining strains were of ovine origin and none of the antisera protected against them.

ACKNOWLEDGMENTS

I am indebted to the following workers for providing the strains of *P. septica* :—

Dr. C. Battelli, Professor de Blieck, Dr. N. H. Casselberry, Señor Julio Conda, Farmaceuticas S.A. Mexico, Dr. W. L. Hindmarsh, Dr. Aage Jepsen, Mr. C. A. McGaughey, Dr. F. C. Minett, Señor Santos Ovejero, Dr. G. S. Schilling, Dr. C. R. Schroeder, Dr. A. W. Stableforth, Signor M. Testa, M. Truche, and Dr. A. Fernandez Zúniga.

I wish to thank Miss J. A. F. Townsend and Mr. D. Doyle for their technical assistance.

REFERENCES

Battelli, C. (1944). *Boll. Soc. Ital. Med. Ig. Trop.*, **3,** 1.
Bringham, G. D., and Rettger, L. F. (1935). *J. inf. Dis.*, **56,** 225.
Bythell, D. W. P. (1945). *Vet. Rec.*, **57,** 289.
Citron, J., and Putz, R. (1907). *Z. Hyg. InfektKr.*, **56,** 145.
Cornelius, J. T. (1929). *J. Path. Bact.*, **32,** 355.
Chamberland and Jouan. (1906). *Ann. Inst. Past.*, **20,** 81.
Edington, J. W. (1930). *J. comp. Path.*, **43,** 239.
Frohböse, H. (1926). *Zbl. Bakt.*, **100,** 213.
Gallagher, B. (1917). *J. Amer. vet. med. Ass.*, **3** (N.S.), 708.
Gochenour, W. S. (1924). *Ibid.*, **65,** 433.
Hutyra, F. (1920). Berl. *tierarztl. Wchnschr.*, **36,** 185.
Jacotot, H. (1940). *Ann. Inst. Past.*, **64,** 56.
Jones, F. S. (1921). *J. exp. Med.*, **34,** 561.
Khalifa, A. B. (1935). *Bull. Off. Int. Epiz.*, **9,** 861. Cited from Battelli (1944).
Klepzow, —. (1904). *Hygiene*, **1,** 221. Cited from Lal (1927).
Kyaw, M. H. (1942). *Vet. J.*, **98,** 3.
Lal, R. B. (1927). *Amer. J. Hyg.*, **7,** 561.
Lignières, J. (1906). *Bull. Soc. cent. Med. vet.*, **83,** 668.
Lovell, R. (1939). *Vet. Rec.*, **51,** 747.
Matsuda, T. (1910). *Z. Hyg. InfektKr.*, **66,** 383.
Miessner and Schern. (1910). *Arch. wiss. prakt. Tierhlk.*, **36,** 208. Cited from Schütze (1929).
Mørch, J. R., and Krogh-Lund, G. (1930). *Z. Hyg. InfektKr.*, **112,** 471.
Newson, J. E., and Cross, Floyd. (1932). *J. Amer. vet. med., Ass.*, **80,** 711.
Pribram, E., and St. Plassaj. (1921). Abst. in *Bull. Inst. Past.*, **19,** 729.
Roderick, L. M. (1922). *J. inf. Dis.*, **31,** 313.
Schirop, H. (1908). *Zbl. Bakt.*, **47,** 307. Cited from Lal (1927).
Schütze, H. (1929). *A. System of Bacteriology*, **4,** 446. H.M. Stationery Office, London ; (1932). *Brit. J. exp. Path.*, **13,** 289 and 293.
Spray, R. S. (1923). *J. inf. Dis.*, **33,** 97.
Tanaka, A. (1926). *Ibid.*, **38,** 421.
Wasserman and Ostertag. (1902). *Mon. prakt. tier.*, **13,** 467.
Webster, L. T. (1925). *J. exp. Med.*, **41,** 571.
Yusef, H. S. (1935). *J. Path. Bact.*, **41,** 203.

[*Received for publication May 5th,* 1947]

# STUDIES ON *C. PYOGENES* ANTITOXIN IN RELATION TO IMMUNITY

## I.—THE PRODUCTION OF *C. PYOGENES* ANTITOXIN IN THE SERUM OF NORMAL DAIRY COWS FOLLOWING THE INOCULATION OF ALUM-PRECIPITATED TOXOID AND OF A COMMERCIAL TOXOID

By

Bernard Weitz

*Ministry of Agriculture and Fisheries, Veterinary Laboratory, Weybridge*

### INTRODUCTION

In view of the importance of *Corynebacterium pyogenes* in animal pathology a series of investigations was begun in 1944 one of which was a study of the extent to which the pathogenic effect of the organism is due to the toxin it produces and the degree of protection afforded against experimental and natural infection by a high level of specific antitoxin in the blood.

Lovell (1937) demonstrated the presence of toxin in filtrates of *C. pyogenes* and the production of antitoxin in rabbits inoculated with the formol toxoid. Subsequently (Lovell, 1941) he noted that whereas cattle gave but a poor response to formal toxoid, sheep reacted well to alum-precipitated toxoid producing a high titre of antitoxin. In a later report on the use of alum-precipitated toxoid in cattle, Lovell (1942) observed that two injections given at an interval of four to six weeks were not sufficient to keep the antitoxin in the blood stream at a reasonably high level for any length of time.

The object of the work reported here therefore was to find the most practicable way to produce and maintain a high antitoxin level in normal cattle. Two preparations were used, an alum-precipitated toxoid and a product marketed as "*C. pyogenes toxoid*" which has been widely used in cattle in the field and will be referred to as "The Commercial Toxoid" (hereafter known as "c.t."). Information was sought on the following points :

1. The system of dosage necessary to produce the maximum of antitoxin as quickly as possible and to maintain it for as long as possible.
2. The antitoxinogenic value of alum-precipitated toxoid compared with that of the commercial toxoid.
3. The nature of the local reaction to injections, if any.

### METHODS

Estimations of the antitoxin content in the blood were made by the *in vitro* haemolytic test described by Lovell (1939) and at each test a standardised control serum was used containing a known amount of antitoxin. The results are expressed as the number

of " x " units of antitoxin per c.c. of serum. The normal content of antitoxin was estimated prior to the inoculation of the experimental animals and subsequently blood samples were drawn at intervals frequent enough to show variations of the antitoxin level. Some 650 blood samples were taken from the thirty cows used for the experiment. The animals were drawn at random from two different herds containing Ayrshire, Friesian and Shorthorn cattle. No unusual incidence of *C. pyogenes* infections had been known to occur in either of these herds.

The *alum-precipitated toxoid* (A.P.T.) was prepared by the method described by Lovell (1939) and consisted of a bacterium-free filtrate of medium in which a highly toxinogenic organism had been grown for 48 hours ; the toxin in the filtrate was converted to toxoid by the addition of formalin and then further concentrated by precipitation with potash alum. The final product was tested in rabbits and gave rise to an average of 80 to 100 " x " units per c.c.

The *" commercial toxoid "* (c.t.) was prepared from cultures in horse meat broth with a large amount of horse meat added and the supernatant fluid, after suitable straining, was formolised. In our hands this product failed to produce antitoxin when tested in rabbits.

## EXPERIMENTAL FINDINGS

### *Initial Response*

The 30 experimental animals were divided at random into six groups, four of which received A.P.T., and two c.t. There were six animals in Group I, four in Group V, and five in each of the others.

*Dosage* : The dose of A.P.T. previously used in the field has been two injections of 10 c.c. given at a ten-day interval and this was taken as the basic dose (Group III, Table I). In Group I a smaller initial dose was added since it was thought that this might enhance the rapid production of antitoxin. It was also useful to know from a practical point of view if a single dose of 20 c.c. would have the same effect as two doses of 10 c.c., and this constituted Group IV. As regards c.t., the recommendation of the makers that doses of 10, 15 and 20 c.c. be given at three-day intervals was adopted in Group V and a comparative group using a similar dosage of A.P.T. was established (Group II). Lastly, a single dose of c.t. (Group VI) was compared with a similar dose of A.P.T. (Group IV).

All inoculations were made intramuscularly in the region of the shoulder or croup.

*Results* : The average antitoxin response for each group will be seen in Table I and Fig. 1.

The response obtained from the injection of A.P.T. was in all cases higher than that produced by the inoculation of c.t. Group I gave the maximum response, *viz.* an average increase of 1229 " x " units of antitoxin.

Groups III and IV both showed a low antitoxin response (200 unit increase) after the injection of two doses of 10 c.c. of A.P.T.

TABLE I

| Group | Material | First injection series | 'x' Units of antitoxin per c.c. | | | Second injection series | 'x' Units of antitoxin per c.c. | | | | Third injection series | 'x' Units of antitoxin per c.c. | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Initial | Maximum | Increase | | Before | Response value | Increase | Comparison with uninoculated controls | | Before | Response value | Increase |
| I | A.P.T. | 5, 10, 10 c.c. at ten- and seven-day intervals | 51 | 1,280 | 1,229 | 10 c.c. | 360 | 413 | +53 | +353 | 10 c.c. | 200 | 1,493 | +1,293 |
| II | „ | 10, 15, 20 c.c. at three-day intervals | 39 | 702 | 663 | 20 „ | 200 | 410 | +210 | +290 | 10 „ | 86 | 1,280 | +1,194 |
| III | „ | 10, 10 c.c. at ten-day intervals | 134 | 352 | 218 | 10 „ | 73 | 480 | +407 | +400 | 10 „ | 200 | 2,086 | +1,826 |
| IV | „ | 20 c.c. | 59 | 258 | 199 | 20 „ | 93 | 653 | +440 | +553 | 10 „ | 93 | 1,660 | +1,567 |
| V | c.t. | 10, 15, 20 c.c. at three-day intervals | 52 | 215 | 163 | 20 „ | 70 | 60 | —10 | —10 | — | — | — | — |
| VI | „ | 20 c.c. | 46 | 60 | 14 | 20 „ | 46 | 46 | 0 | 0 | — | — | — | — |

at a ten-day interval and a single dose of 20 c.c. respectively. The response in Group II was intermediate. Group V which received the commercial toxoid in the dosage recommended by the manufacturers showed only a small increase in antitoxin and as compared with the response obtained to a similar dosage of A.P.T. (Group II) there was a difference of 500 units to be noted in favour of the latter. A single dose of 20 c.c. of c.t. had no appreciable effect on the level of antitoxin in the blood.

*Second response*

In each group, half of the experimental animals were reinoculated with the same material as soon as the level of antitoxin in the blood fell towards normal, the dose given being the same as the last one in the previous series of injections. This second stimulus was given 86 days after the beginning of the experiment by which time the level of antitoxin was almost back to normal in Groups III, IV, V and VI and was dropping in the other groups. The remaining animals in each group were left as controls. The results are shown in Table I and Fig. 1.

*Third response*

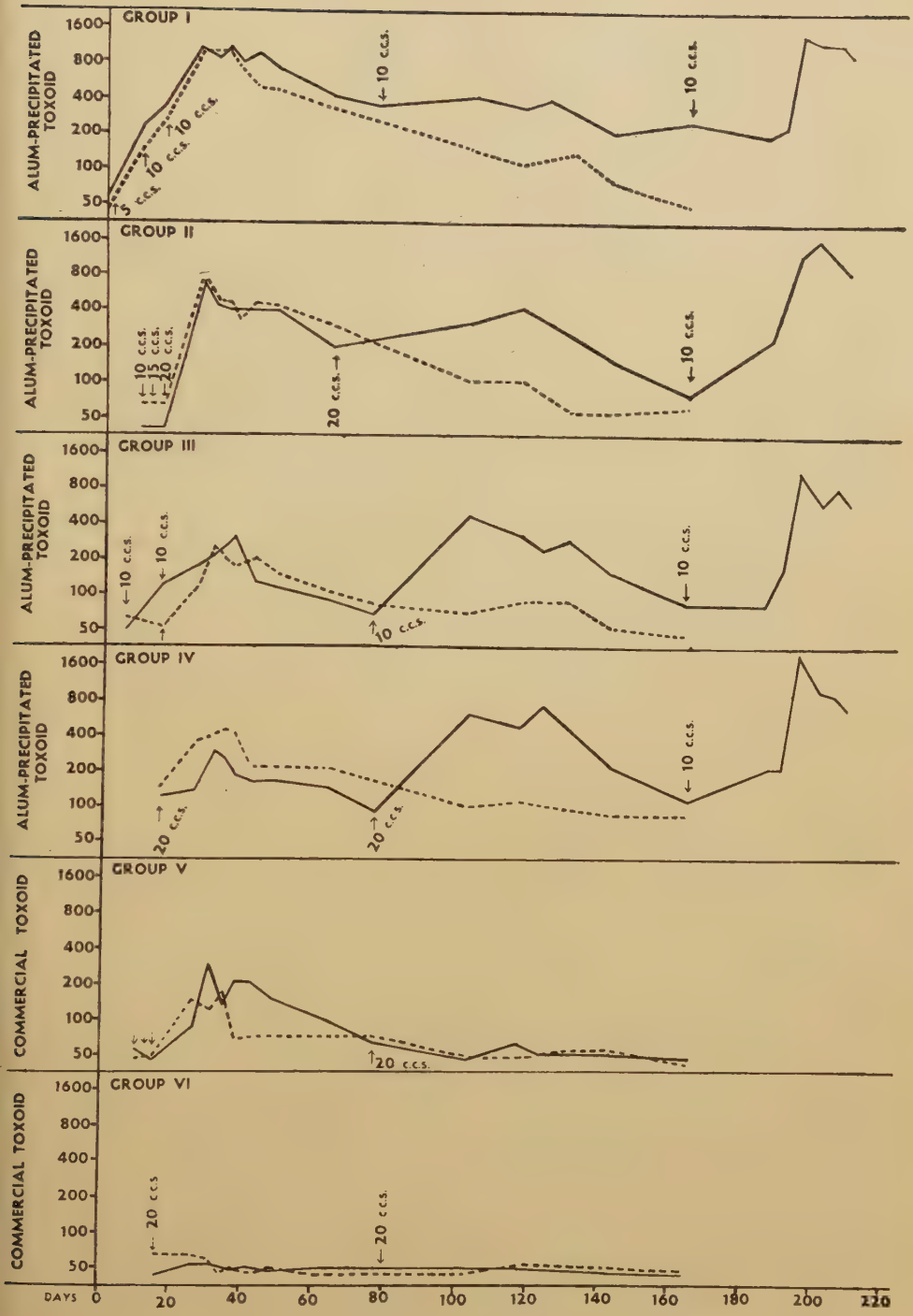
In view of their response to the second stimulus it was decided to inoculate the same cows a third time. The intention was to do this, as on the previous occasion, when the antitoxin was falling towards its normal level, but the injections were unavoidably delayed until 160 days after the beginning of the experiment by which time the titre had reverted approximately to normal in all groups. A dose of 10 c.c. of A.P.T. was given intramuscularly as before. There seemed to be no need to continue with the animals which had been receiving c.t. and Groups V and VI were therefore excluded. *Results* : In all animals a response was obtained which compared most favourably with any previously observed. There was no significant difference in the results for the various groups and they appeared to be independent of previous responses (Table I : Fig. 1).

## DISCUSSION

The chief object of this work was to devise a practical method by which the antitoxin level in the blood of cattle could be suitably raised and maintained at its maximum for as long a period as possible. All groups which received A.P.T. showed an equivalent response following the third series of inoculations at 160 days. The method of choice for the production of a high level of antitoxin would therefore be the one used in Group IV which consists of the minimum number of injections, namely, three injections of 20, 20 and 10 c.c. at 86- and 160-day intervals.* This method might

* A more practical method of dosage of 5, 10 and 10 c.c. A.P.T. given at two-monthly intervals has since been tried with equally good results and will be reported in a later publication.

FIG. 1


GROUP I
ALUM-PRECIPITATED TOXOID
1600
800
400
200
100
50
5 c.c.s.
10 c.c.s.
10 c.c.s.
10 c.c.s.
10 c.c.s.
10 c.c.s.
GROUP II
ALUM-PRECIPITATED TOXOID
10 c.c.s.
15 c.c.s.
20 c.c.s.
20 c.c.s.
10 c.c.s.
GROUP III
ALUM-PRECIPITATED TOXOID
10 c.c.s.
10 c.c.s.
10 c.c.s.
10 c.c.s.
GROUP IV
ALUM-PRECIPITATED TOXOID
20 c.c.s.
20 c.c.s.
10 c.c.s.
GROUP V
COMMERCIAL TOXOID
20 c.c.s.
GROUP VI
COMMERCIAL TOXOID
20 c.c.s.
20 c.c.s.
DAYS 0 20 40 60 80 100 120 140 160 180 200 220

be useful in field trials against Summer Mastitis when it is desired to obtain a high titre of antitoxin at a certain time of the year. The first dose could be given in the winter followed by an intermediate one in March or April, and a final one in late June, thus obtaining the maximum titre during July. It is even doubtful, at this stage of the work, whether the intermediate dose is of any real value. When, however, a more rapid rise in antitoxin is required, the method used for Group I would be applicable, *viz.* 5, 10 and 10 c.c. at seven- and ten-day intervals, and would give an antitoxin titre of approximately 1,000 " x " units per c.c. within two weeks after the last injection. The level could then be maintained by further injections of 10 c.c. at two-monthly intervals.

It will be seen from Table I and Fig. 1, that when A.P.T. is used the quality of the second response seems to depend on the height of antitoxin level obtained in the initial response and is inversely proportional to it. Thus in Groups I and II which gave a good response in the first instance, the second series of inoculations given on the 86th day, produced only a slight rise of antitoxin. Conversely, a good response was obtained in Groups III and IV which had shown only a poor initial response. The highest average level of antitoxin produced in the cows was somewhat below 2,000 " x " units per c.c. This figure was never exceeded even at the peaks of the various responses and seems to be the maximum obtainable by the methods used. It will be noted that though the amount of toxoid given in Group II was more than double that which was used in Group IV no further significant increase of antitoxin was obtained at 167 days. (The average of the four groups at this time was 1,176 " x " units per c.c.). These observations suggest that the limiting factor for antitoxin production may have been connected with the animal rather than with the antigen.

The " commercial toxoid " appeared to have low antitoxinogenic properties in cattle, however used. The lack of response may be explained by (1) the absence of concentration of toxoid in the final product (2) the failure to grow the organism in the most suitable medium for toxin production (Lovell, 1941), and (3) the absence of alum in the inoculum.

The use of toxoid or vaccine for the prevention of *C. pyogenes* infections forms the subject of the succeeding paper (Weitz and Langridge, 1947).

Finally, it should be noted the inoculations resulted in no apparent ill effects on the health of normal cows. Milk yields were checked at both farms and showed no change whatever when compared with uninoculated animals in the herds, even in spite of the repeated bleedings which were made. A total of 90 separate injections were given and only on two occasions was a slight transient swelling observed. Three animals were examined *post-mortem* and no trace of the injections could be seen.

SUMMARY

A good antitoxin titre was obtained by the use of *C. pyogenes* A.P.T. when given in a dose of 5, 10, and 10 c.c. at ten- and seven-day intervals. Bi-monthly rejections of 10 c.c. helped to maintain the level of antitoxin.

Various doses were tried and all gave a satisfactory result after three series of inoculations given at 86- and 160-day intervals.

It appeared a maximum level of antitoxin production in cattle was reached.

The " commercial toxoid " was not antitoxinogenic.

No ill effects resulted from the injections.

ACKNOWLEDGMENTS

I am deeply grateful to Dr. W. S. Gordon, director of the A.R.C. Field Station at Compton, and to Mr. Hunter-Smith, Principal of the Hertford Agricultural College, for allowing me to use their herds for this experiment.

I am also grateful to Dr. Stableforth for his help and guidance throughout the experiment and in the preparation of this paper.

REFERENCES

Lovell, R. (1937). *J. Path. Bact.*, **45**, 399 ; (1939). *Ibid.*, **49**, 329 ; (1941). *Ibid.*, **52**, 295 ; (1942). *Proc. r. Soc. Med.*, **36**, 151.

Weitz, B., and Langridge, R. (1947). *J. comp. Path.*, **57**, 286.

[*Received for publication May 27th,* 1947]

# STUDIES ON *C. PYOGENES* ANTITOXIN IN RELATION TO IMMUNITY

## II.—THE EFFECT OF ALUM-PRECIPITATED TOXOID AND VACCINE ON THE ARTIFICIAL INFECTION OF THE UDDER OF THE EWE WITH *C. PYOGENES*

By

Bernard Weitz and R. Langridge *

*Ministry of Agriculture and Fisheries, Veterinary Laboratory, Weybridge*

### INTRODUCTION

In a previous paper (Weitz, 1947) a suitable method for the production of a high level of antitoxin in cattle by means of alum-precipitated toxoid was described. The object of the work reported here was to determine the degree of protection which this method can afford against experimental infection of the udder with *C. pyogenes* compared with that conferred by formalinised vaccine which produces little rise in antitoxin level. Sheep were used for the purpose because they are known to be susceptible to udder infection with *C. pyogenes* and can more easily be accommodated in fairly large numbers under experimental conditions than cattle. Moreover, Lovell (1941) has shown that they give a particularly good antitoxin response to alum-precipitated toxoid.

### MATERIALS AND METHODS

*Alum-precipitated toxoid.* This was prepared as described by Lovell (1939) and standardised by the amount of antitoxin produced in rabbits.

*Vaccine. C. pyogenes* grown on sheep blood agar in Roux flasks at 37° C. for three days was suspended in Ringer's solution to an opacity equivalent to that of Brown's tube No. 2 and 0·4 per cent. formalin was added ; the product was incubated for two days at 37° C. and tested for sterility on blood agar plates and by animal inoculation.

*Blood.* Nine samples of blood were taken from each ewe, *viz.*, on the days on which inoculations of toxoid or vaccine were made, six days following the last inoculation, on the day on which the udders were infected, and at weekly intervals thereafter until the end of the experiment. *C. pyogenes* antitoxin content was estimated by the *in vitro* method as described by Lovell (1939).

*Milk.* Milk samples were drawn from each half of the udder before infection and every three days subsequently. Films were made and stained by Leishman's method and the milk was cultured in duplicate on blood agar plates which were incubated for 48 hours at 37° C. Alterations in the character of the milk were noted at the time of collection.

*Material for Infection.* The strain of *C. pyogenes* used for the test infection of the ewes was obtained from a recent case of purulent endometritis in a cow. A large dose was at first needed to produce permanent udder infection in ewes but after two passages its virulence was much

* Centenary Research Fellow, Royal (Dick) Veterinary College, Edinburgh.

enhanced. In the main experiment each ewe was given two doses : a comparatively small one (115,000 viable organisms) in the left half of the udder, and a larger one (x $10^4$) in the right half. These doses which were prepared from a 48 hr. growth of the passage strain on sheep blood agar, suspended in Ringer's solution containing 1 per cent. broth, were injected by means of a teat syphon attached to a hypodermic syringe and the udder was massaged lightly.

## PLAN OF THE EXPERIMENTS

Eighty recently lambed ewes in full lactation were used and were run with their lambs until they were due to be tested for immunity.

Six ewes were taken for preliminary titration of the infective dose required for the test and the remaining 74 were divided into four groups as follows :—

Eighteen received *C. pyogenes* alum-precipitated toxoid in doses of 5, 10 and 10 c.c. at ten-day intervals.

Eighteen received *C. pyogenes* vaccine in doses of 2·5, 5 and 5 c.c. at ten-day intervals.

Eighteen were given vaccine as well as toxoid in the doses mentioned above.

Twenty were kept as controls.

All injections of toxoid and vaccine were made into the muscles of the croup. Thirteen days after the last injection cultures of *C. pyogenes* were inoculated *via* the teat canal in all ewes except three or four in each group. These were left uninoculated in order to follow the normal course of milking in ewes following weaning and to assess any difference in antitoxin content produced by infection. At this time the lambs were removed from their mothers.

## RESULTS

### *Antitoxin Response*

The response to the injection of toxoid and of vaccine is shown in Table I. The figures indicate the average number of " x " units per c.c. for each group at various times. The inoculations of alum-precipitated toxoid produced a marked increase in antitoxin both when used alone and when inoculated in conjunction with vaccine, the peak antitoxin titre produced being an average of about 13,000 units in the first case and nearly 8,000 in the other. It would appear that the third inoculation failed to provide a further stimulus in antitoxin production but the peak possibly occurred between the third and fourth bleedings. No response is evident in those sheep which received only vaccine or in the control sheep. At the time of test infection the content of antitoxin in the groups which had been inoculated with toxoid was still very high and approximated 2,000 units per c.c., while in the sheep which had received vaccine only and in the control sheep the average antitoxin content was in the region of 100 units per c.c. which can be regarded as roughly the normal level in sheep. Following the test infection on the 35th

day an appreciable rise in antitoxin content appeared in all infected animals. The non-infected ewes showed no further increase in antitoxin content and in most cases fell as seen in Table I, Columns (*b*).

In the toxoid group, the response in antitoxin following infection seems to be delayed as compared with other groups. This apparent delay may be due to the relatively slower onset of disease (see Table II and Fig. 1) following the inoculation of the infective dose in this particular group.

TABLE I

AVERAGE ANTITOXIN CONTENT IN 'x' UNITS PER C.C. OF SERA OF SHEEP IN EACH GROUP

| *Days* | *Toxoid* | | *Toxoid and Vaccine* | | *Vaccine* | | *Controls* | |
|---|---|---|---|---|---|---|---|---|
| | *a* | *b* | *a* | *b* | *a* | *b* | *a* | *b* |
| 0 | 60 | 56 | 66 | 54 | 44 | 81 | 84 | 83 |
| 13 | 1,890 | 440 | 1,453 | 800 | 37 | 56 | 28 | 123 |
| 22 | 12,960 | 8,860 | 7,786 | 5,653 | 224 | 326 | 212 | 240 |
| 29 | 8,531 | 1,560 | 2,048 | 2,506 | 55 | 167 | 118 | 200 |
| 35* | 2,772 | 560 | 1,720 | 1,306 | 96 | 373 | 138 | 132 |
| 41 | 2,101 | 1,280 | 1,813 | 1,600 | 126 | 126 | 132 | 106 |
| 48 | 852 | 360 | 1,850 | 786 | 389 | 93 | 557 | 70 |
| 56 | 7,127 | — | 1,165 | 140 | 4,707 | — | 1,897 | — |
| 66 | 6,210 | 380 | 5,840 | 320 | 1,993 | 100 | 4,248 | 200 |
| Total number of sheep in each Group | 16 | 2 | 15 | 3 | 15 | 3 | 16 | 4 |

*a* = Sheep which were infected on 35th day.
*b* = Sheep which were not infected.
* = Infection of animals in columns (*a*).

### *The Experimental Disease*

There was little difference in the course of the disease produced by the small dose of organisms and that produced by a much larger dose, but a difference in the incidence of disease was observed.

*Clinical symptoms.* Twenty-four hours after infection, the affected half of the udder became tense and somewhat distended. (This was observed also in some of the non-inoculated udders and may well have been the result of weaning). Shortly afterwards the milk became sticky, less homogenous than normal, and in most cases greenish and within three to seven days a well defined mastitis characterised by a purulent secretion developed. Nodular fibrosis and gradual shrinking of the organ followed. In a number of cases abscesses formed in the udder and ruptured, discharging yellow pus containing *C. pyogenes* in pure culture. The diseased halves tended to dry off and in a proportion of cases no secretion was obtainable after two or three weeks.

The general health of the ewes was not seriously affected ; feeding was normal and there were no signs of distress apart from local

FIG. 1

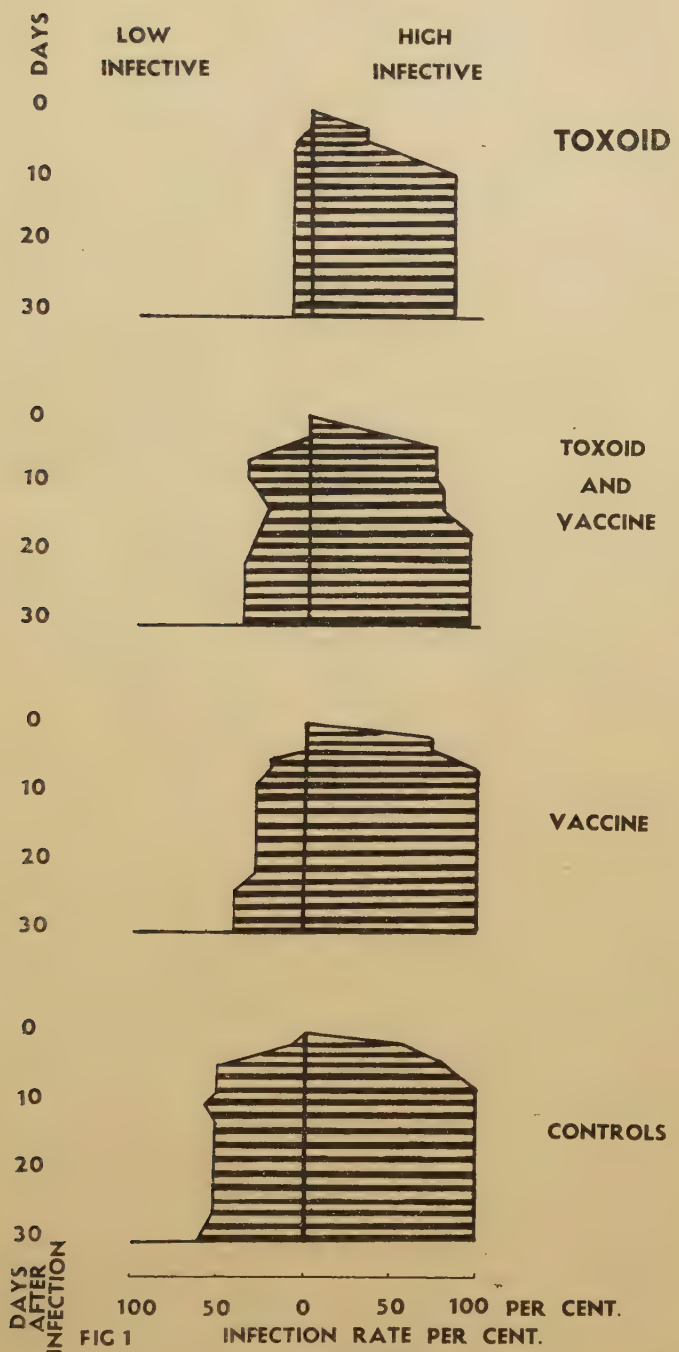



FIG 1

FOLLOWING
LOW AND HIGH INFECTIVE DOSES

pain or discomfort, especially when the udder was manipulated. By the end of the experiment most of the animals appeared in normal health although the local disturbance persisted.

*Bacteriological examination.* *C. pyogenes* was isolated from typical cases throughout the course of the disease, in relatively small numbers at first but in great abundance once the purulent stage was reached.

*Complications.* Superimposed infections of a haemolytic staphylococcus occurred in several instances and were characterised by the very acute symptoms typically associated with this form of mastitis in sheep and the death rate was high. These cases are included in the total of infected animals in Table II, the figures in brackets showing the number of living ewes which at any given time were infected with haemolytic staphylococcus in addition to *C. pyogenes*.

Infection with *Proteus* bacilli was encountered in five cases, in all of which it occurred subsequent to a primary infection with *C. pyogenes*. In these circumstances it was possible to isolate the latter from the milk if steps were taken to prevent swarming of the *Proteus* colonies by the use of nutrient blood agar made with 4·2 per cent. New Zealand agar. This method was originally suggested by Hayward and Miles (1943) who, however, used 6 to 8 per cent. agar (presumably Japanese agar). The present modification gives a medium of the same consistency which, owing to the greater purity of New Zealand agar, does not need clearing; this is an important point since clearing by filtration is impossible with this concentration of agar. It was not clear to what extent, if any, the *Proteus* bacilli infected the mammary tissue or whether the infection was localised to some lesion in the teat canal, but the organisms were recovered invariably from such halves. No additional symptoms due to the superimposed infection were observed.

### COMPARISON OF RESULTS IN THE VARIOUS GROUPS

The results are expressed in terms of the number of halves which were severely and more or less permanently affected. By this is meant those which showed acute symptoms of mastitis as described in the previous section and yielded milk which was altered in nature and contained innumerable *C. pyogenes*. Slight transient infections which did not give rise to acute symptoms were not included in totalling the results.

In Table II the infection data at various intervals after inoculation of the test infective material are classified according to the method of immunization, if any, and size of the infective dose. The numerator expresses the number of infected halves while the denominator shows the total number of halves concerned. Variations in this figure are due to deaths or losses from causes other than *C. pyogenes* infection. Once infection was established in a particular case this was totalled right through the experiment even though the quarter had become dry in some cases and no sample was obtained.

Little difference can be observed between the various groups as regards the high infective dose. In those groups which received toxoid, the incidence of infection rose more gradually and did not quite reach 100 per cent. but showed little significant difference from the vaccine or control groups.

With the lower infective dose, there is a clearly marked difference in the rate of infection between the various groups. About 50 per cent. of sheep in the control group became permanently infected in the left half of the udder. In the groups which received vaccine, or vaccine and toxoid, a slight reduction in incidence can be observed, *viz.*, from 50 per cent. to approximately 35 per cent. There is, however, a striking reduction in the number of halves infected among the sheep which had received toxoid alone ; only one case of permanent infection was noted out of 12 sheep involved (8·3 per cent.) and this sheep also showed a superimposed infection of *Proteus* bacilli. One other animal in this group showed a mild transient infection in the milk from the left half, but at no time were clinical changes noticeable in the udder nor was the milk altered in character.

TABLE II

INCIDENCE OF MASTITIS IN SHEEP FOLLOWING EXPERIMENTAL INFECTION WITH *C. pyogenes*

| *Group* | *Infection dose* | *Number of days after infection* | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 2 | 4 | 6 | 8 | 10 |
| Toxoid | High dose | 0 | 5 | 4 | 6 | 10 | 11 |
| | | — | — | — | — | — | — |
| | | 15 | 15 | 13(1) | 12 | 12 | 12 |
| | Low dose | 0 | 0 | 1 | 1* | 1* | 1* |
| | | — | — | — | — | — | — |
| | | 16 | 16(2) | 14(3) | 12(1) | 12(1) | 12(1) |
| Toxoid and vaccine | High dose | 0 | 5 | 10 | 10 | 10 | 11 |
| | | — | — | — | — | — | — |
| | | 15 | 13(2) | 14(1) | 14 | 14 | 14 |
| | Low dose | 0 | 0 | 2 | 5 | 5 | 4 |
| | | — | — | — | — | — | — |
| | | 15 | 15(2) | 11(1) | 13 | 13 | 13 |
| Vaccine | High dose | 0 | 10 | 10 | 14 | 14 | 14 |
| | | — | — | — | — | — | — |
| | | 14 | 14 | 14 | 14 | 14 | 14 |
| | Low dose | 0 | 0 | 3 | 3 | 4 | 4 |
| | | — | — | — | — | — | — |
| | | 15 | 15 | 15 | 14 | 12(1) | 12(1) |
| Controls | High dose | 0 | 8 | 12 | 13 | 14 | 14 |
| | | — | — | — | — | — | — |
| | | 16 | 15 | 15 | 14 | 14 | 14 |
| | Low dose | 0 | 1 | 6 | 6 | 6 | 7 |
| | | — | — | — | — | — | — |
| | | 16 | 16 | 12 | 12 | 12 | 12 |

(*Continued at top of p.* 292)

| Group | Infection dose | Number of days after infection | | | | | |
|---|---|---|---|---|---|---|---|
| | | 13 | 16 | 21 | 24 | 28 | 31 |
| Toxoid | High dose | 11 | 11 | 11 | 11 | 11 | 11 |
| | | — | — | — | — | — | — |
| | | 12 | 12 | 12 | 12 | 12 | 12 |
| | Low dose | 1* | 1* | 1* | 1* | 1* | 1* |
| | | — | — | — | — | — | — |
| | | 12(1) | 12(1) | 12(1) | 12(1) | 12(1) | 12(1) |
| Toxoid and vaccine | High dose | 11 | 12 | 13 | 13 | 13 | 13 |
| | | — | — | — | — | — | — |
| | | 14 | 14 | 14 | 14 | 14 | 14 |
| | Low dose | 3 | 4 | 5 | 5 | 5 | 5 |
| | | — | — | — | — | — | — |
| | | 13 | 13 | 13 | 13 | 13 | 13 |
| Vaccine | High dose | 14 | 14 | 14 | 14 | 14 | 14 |
| | | — | — | — | — | — | — |
| | | 14 | 14 | 14 | 14 | 14 | 14 |
| | Low dose | 4 | 4 | 4 | 4 | 4 | 4 |
| | | — | — | — | — | — | — |
| | | 12 | 12 | 12 | 12 | 12 | 12 |
| Controls | High dose | 14 | 14 | 14 | 14 | 14 | 14 |
| | | — | — | — | — | — | — |
| | | 14 | 14 | 14 | 14 | 14 | 14 |
| | Low dose | 6 | 6 | 6 | 6 | 7 | 7 |
| | | — | — | — | — | — | — |
| | | 12 | 12 | 12 | 12 | 12 | 12 |

Numerator indicates number of " infected " ewes.
Denominator indicates total number of ewes involved.
Figures in brackets show number of sheep suffering from haemolytic staphylococcus infection.

* Milk infected with *Proteus* bacilli.

## DISCUSSION

Ewes are readily susceptible to experimental infection of the udder with *C. pyogenes* and show a characteristic type of mastitis. They give a very good antitoxin response to toxoid but none to vaccine.

The results of the test infection suggest that toxoid may be of some value in the prevention of infection but no evidence in favour of vaccine was observed. Although almost the same amount of antitoxin was produced whether toxoid was used alone or in conjunction with vaccine, the results of infection show a difference in favour of toxoid alone. It is not clear what phenomenon is concerned, but further suggestions of the unfavourable effect of vaccine when used with toxoid have been noted on other occasions. (Unpublished work by B.W.).

A significant degree of protection was conferred by toxoid against the low infective dose, but nothing appreciable against the higher. The latter appears too great for the testing of prophylactic substances.

SUMMARY

Artificial infection of the udder of ewes with *C. pyogenes* produces a typical mastitis.

A high level of antitoxin is produced in sheep following inoculations with A.P.T.

Considerable protection against artificial infection of the udder of ewes is afforded by A.P.T. when the infecting test dose is such that it produces 50 per cent. infection in the controls. When a higher infecting dose was used no protection was evident in sheep.

ACKNOWLEDGMENTS

We are grateful to Dr. A. W. Stableforth for his help and advice in this work and in the preparation of this paper.

REFERENCES

Hayward, N. J., and Miles, A. A. (1943). *Lancet*, **2**, 116.
Lovell, R. (1939). *J. Path. Bact.*, **49**, 329 ; (1941). *Ibid.*, **52**, 295.
Weitz, B. (1947). *J. comp. Path.*, **57**, 279.

[*Received for publication May 27th*, 1947]

# THE MAINTENANCE OF CATTLE UNDER CONDITIONS OF TSETSE FLY INFESTATION IN THE FIELD

By
J. K. H. Wilde,
*Veterinary Research Laboratory, Mpwapwa, Tanganyika*

## INTRODUCTION

The experiments reported in this paper were part of a provincial scheme for the removal of natives and their cattle from certain heavily eroded areas of the Kondoa-Irangi district, to the extensive potential grazing areas on the edge of the Masai plain which borders on the Bereku ridge along the Chungia-Itundwe-Cheke-Filimo-Kisese line. Bush clearance is being carried out by the Department of Tsetse Research in this area but it will be a long time before it is completely freed of fly. As the clearing proceeds, however, the fly density is decreasing and areas of potential grazing, which are excellent and plentiful, are increasing.

The herds enumerated below were chosen to ascertain whether it would be economically sound to introduce cattle into the area, and, by means of drugs, to maintain them satisfactorily even before the tsetse fly had been completely eliminated. At the same time, it was hoped that useful information regarding the use of the two drugs selected, *viz.*, 897, a derivative of phenanthridinium chloride (Hornby *et al.*, 1943), and Stibophen, would be forthcoming. One hundred and fifty-three cattle were obtained and were divided into herds which were disposed as follows (see sketch map).

Herd A contained 11 animals, several of which were cows, kraaled at Chungai and grazed in the Chungai area.

Herd B contained 31 animals, kraaled at Msiya Hill about one mile east of Chungai, and grazed in an area at the foot of Msiya Hill near the edge of tsetse infested bush. (*G. morsitans*).

Herd C contained 46 animals kraaled at Itundwe two miles north-west of Chungai and herded in the vicinity of the kraal. This herd was divided at random into three groups of 15, 16 and 15 animals respectively (Nos. *a*, *b* and *c*); all three groups, however, were kraaled and grazed together.

Herd D contained 25 animals, kraaled and grazed at Cheke about one and half miles north of Itundwe at the foot of the western escarpment.

Herd E contained 40 animals, kraaled and herded on the open mbuga (swamp) in a corridor free from bush and about four and a half miles east of the escarpment.

At the beginning of the experiment in September, 1944, all the animals were poorly developed adults and few were estimated to weigh more than 2 cwt. Although they had come from a "fly free" area, they had been kept at Chungai for two or three weeks before the experiment started. Some of them may, therefore, have

become infected before treatment was commenced. For this reason a modification in the interpretation of the results was introduced (see below).

## METHOD

All the animals were subjected to blood-slide examinations every fortnight and treatments were carried out as follows :—

Herd A (Chungai)—Animals showing trypanosomes in their blood were treated with Stibophen twice at a week's interval.

Herd B (Msiya Hill)—Animals showing trypanosomes in their blood were given one dose of 897.

Herd C (Itundwe)—All animals in group (*a*) received a dose of Stibophen once monthly, irrespective of their showing trypanosome infection or not.

All animals in group (*b*) received 897 monthly, irrespective of their showing trypanosome infection or not.

Only infected animals in group (*c*) were treated and these received one dose of 897 after each fortnightly blood slide which proved positive.

Herd D (Cheke) and Herd E (Mbuga)—These were treated with 897 only after giving a positive blood slide.

## ASSESSMENT OF RESULTS

The results of the blood-slide examinations are given in tabular form and each herd will be discussed separately. Before proceeding to an analysis, however, the manner of assessment of the incidence of trypanosomiasis must be explained. As there is no means of determining under conditions of tsetse infestation whether the fresh appearance of trypanosomes in the blood represents a fresh infection or a relapse to a former one, arbitrary rules were adopted.

In the case of *T. congolense* and *T. vivax* infections, if one month elapsed between positive films, *i.e.*, two successive negative examinations, the second manifestation was considered as a fresh infection. Trypanosomes appearing in the first month of examination were disregarded as they might well have been picked up before the animals were brought into the experiments. It is thus possible that by this method a number of relapses have been counted as fresh infections and *vice versa*. An endeavour, however, has been made to deal with the results for each animal in exactly the same way so as to minimise the error or disproportion of the final picture.

With regard to *T. brucei* it was thought that a different view must be taken. This parasite may exist for long periods in the blood of a bovine without causing any obvious pathological changes, disappearing and reappearing from time to time in the peripheral circulation. Neither of the drugs used in this experiment has proved to be satisfactory against *T. brucei* and thus, for reasons stated below, treatment, although given when this trypanosome appeared in the blood, was not expected to be effective. It was decided that, in

considering the results of the trypanocidal effect of the drugs under examination, the incidence of *T. brucei* must be ignored. This decision was strengthened by the fact that in some cases the diagnosis of *T. brucei* was probably inaccurate and may have been confused with *T. simiae*. While *T. brucei* was recorded it is not included in the calculations : nevertheless its presence is useful as it helps to indicate the density of trypanosome-infected tsetse flies.

At the end of the experiment, which occupied 14 months, all the cattle involved were in excellent condition and had increased considerably in weight. A few had died of intercurrent disease but none of these deaths could be attributed either to trypanosomiasis or to the use of the drugs. No permanent lameness resulted from the injections although some animals had received monthly treatment (Herd C, groups *a* and *b*). A few of those which received regular injections of 897 into the gluteal mass, showed a little induration of the muscles at the site of injection. One, in which a swollen buttock persisted, was slaughtered for examination and a large " cold " abscess in the muscles was found. During the course of treatment with 897, temporary swelling of the buttock with occasional lameness often occurs but such cases generally recover rapidly. When the injections are given carefully, the tissue reaction is very slight.

## SUMMARY OF RESULTS

Herd A.—During the period of the experiment ten of the cattle (90·9 per cent.) became infected, one of them five times. The average number of infections was 1·73 per beast. The incidence of *T. brucei* was high. Treatment of infected animals comprised two doses of Stibophen at seven days interval.

Herd B.—In this group of 29 cattle, out of the original 31, 23 (79·3 per cent.) became infected and were treated with a single dose of 897. The average number of infections per beast was 1·72. The incidence of *T. brucei* was high.

Herd C.—This herd was divided into three equal sections with the object of determining whether or not Stibophen and/or 897 have any prophylactic effect against *T. congolense* and *T. vivax*. The following results were obtained :—

Group (*a*).—Nine out of 14 animals (64·3 per cent.) were infected. The average number of infections per beast was 1·0.

Group (*b*).—Four out of 14 animals (28·6 per cent.) were infected. The average number of infections per beast was 0·44.

Group (*c*).—Eight out of 15 animals (53·3 per cent.) were infected. The average number of infections per beast was 0·87.

Herd D.—Twenty-two out of 25 animals (88 per cent.) became infected and were treated with 897. The average number of infections per beast was 1·45. The incidence of *T. brucei* was high.

Herd E.—Seventeen out of 38 animals (44·7 per cent.) were

infected and the average number of infections per beast was 0·68. They were treated with 897.

Throughout the whole experiment, 166 fresh infections with *T. congolense* and *T. vivax* were diagnosed and 862 doses of trypanocidal drugs were administered. There were 576 inoculations of drug 897 and 286 of Stibophen. The number of injections given

TABLE I

COMPOSITE TABLE OF RESULTS*

| Herd | Total no. of animals | Animals infected | Nos. of infections and animals infected | | | | | | Total no. of infections | Average no. of infections per animal |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 5 | 4 | 3 | 2 | 1 | 0 | | |
| A. Chungai | 11 | 10 (90·9%) | 1 | — | 1 | 3 | 5 | 1 | 19 | 1·73 |
| B. Msiya Hill | 29 (†2 of original herd died) | 23 (79·3%) | — | 3 | 4 | 10 | 6 | 6 | 50 | 1·72 |
| C. Itundwe Group (a) | 14 (†1 of original herd died) | 9 (64·3%) | — | — | 1 | 3 | 5 | 5 | 14 | 1·0 |
| Group (b) | 14 (†2 of original herd died) | 4 (28·6%) | — | — | — | 2 | 2 | 10 | 6 | 0·44 |
| Group (c) | 15 | 8 (53·3%) | — | — | 1 | 3 | 4 | 7 | 13 | 0·87 |
| D. Cheke | 25 | 22 (88%) | — | — | 2 | 12 | 8 | 3 | 38 | 1·45 |
| E. Mbuga | 37 (†3 of original herd died) | 17 (44·7%) | — | — | 2 | 4 | 12 | 19 | 26 | 0·68 |

* The original detailed tables are too large for publication.

† None of the eight animals which died during the experiment died of trypanosomiasis nor were their deaths attributable to the use of the drugs. The deaths were due to other diseases, *e.g.*, Anthrax, East Coast Fever, Blackquarter. These animals were disregarded in the assessment of the results.

was considerably in excess of those necessary for *T. congolense* and *T. vivax*, since in order to avoid confusion infections with *T. brucei* were also treated. As 200 inoculations against *T. brucei* were made, the number of doses for *T. congolense* and *T. vivax* which may be considered as " necessary " doses was 662 : 392 of these, administered to 28 cattle, were used prophylactically. The figures are summarised in Table II.

TABLE II

| No. of doses | No. of cattle | No. of doses per beast for 14 months | No. of doses per beast for 12 months |
|---|---|---|---|
| Necessary therapeutic 270 | 122 | 2·21 | 1·89 |
| „ prophylactic 392 | 28 | 14 | 12 |

It is interesting to note that the incidence of trypanosomiasis, shown in the records of the experiments, is very closely related to the incidence of tsetse flies in the same areas as determined by the Tsetse Research Department.

## DISCUSSION

Cattle in very poor condition were introduced into an area of good grazing in which there was a medium tsetse-fly infestation. It was shown conclusively that some of these flies were carrying trypanosomes by the fact that large numbers of the animals became infected with *T. congolense, T. brucei* and *T. vivax*.

It was impracticable to leave any of the infected cattle untreated as controls, but experience in Tanganyika has shown that had this been done many of them would have died. As the result of treatment the cattle not only maintained condition, but thrived noticeably and none died from trypanosomiasis. The drugs used were 897, a member of the phenanthridinium group, and Stibophen, the British equivalent of the antimonial drug Antimosan. Although statistical treatment of the results does not show a marked difference in the effect of these two drugs, an impression was gained that 897 was the more effective.

In the subsidiary experiment with herd C it appears on first examination that a marked difference resulted between (1) the group treated prophylactically with Stibophen (group *a*) and that similarly treated with 897 (group *b*); and (2) the group treated prophylactically with 897 (group *b*) and the control group treated therapeutically with 897 (group *c*).

The results in group (*a*) are not as favourable as those in the control group (*c*). In the latter, however, 897 was used for treatment of animals when they became infected in order to prevent the deaths of infected animals. If 897 has a prophylactic effect, it may have influenced the rate of infection in group (*c*). This finding may, therefore, invalidate to a certain extent the value of this group as controls, but they have to be used for this purpose as they give an infection rate which approaches nearest to that expected in untreated cattle under the same conditions.

Statistical treatment of the data for this herd fails to reveal any real significance between the groups (*a*) and (*b*) on the one hand, and group (*c*) on the other. Application of the Chi Square test, Yates modification for small samples (Mainland, 1938) shows that samples with a proportion of non-infected to infected animals as high or higher than that of group (*b*) could be obtained by normal sampling variation of untreated animals, in more than five times out of 100. Thus, although this cannot be considered as really significant, it does show a tendency towards significance and that the drug 897 might have decreased the rate of infection if only slightly. This tendency might have been more marked had it been possible to use completely untreated controls.

Table II shows without doubt that prophylactic treatment, even were it fully effective, is more expensive than therapeutic treatment when applied to cattle living permanently in a tsetse-infested area. For practical purposes, if cattle are to be maintained by the use of drugs, treatment after infection would be the only economical method. It need not be associated with the regular, laborious routine slide examination of all animals as was the case in the experiment, but could be applied to those showing clinical symptoms of trypanosomiasis. Nevertheless, periodic slide examinations would be advisable in order to detect and treat any sub-clinical carriers.

## CONCLUSIONS

One hundred and forty-five cattle kept for 14 months in an area infested with tsetse-fly received treatment of one kind or another with either 897 or Stibophen. They thrived and none was lost either from trypanosomiasis or the effects of the drugs.

The incidence of trypanosomiasis in the various herds in the experiment was demonstrated and the close correlation between this and the incidence of tsetse flies, as determined independently, was noted.

A subsidiary experiment designed to ascertain if 897 and/or Stibophen had any prophylactic value indicated a possibility in the case of 897 but none for Stibophen. Statistical treatment of the data showed, however, that under the conditions of the experiment this " prophylactic " value was very small, if it existed at all. ,

It is concluded that cattle can be kept in areas of medium tsetse infestation provided suitable chemotherapeutic treatment is applied. Treatment of cattle which show clinical signs of trypanosomiasis is advocated rather than any attempts at prophylaxis.

## ACKNOWLEDGMENT

I wish to express my appreciation of the valuable assistance rendered to me by the Acting Director of Tsetse Research, Mr. Napier Bax and by his staff, especially Mr. H. M. Lloyd and Mr. A. G. M. du Frayer. Also I thank Mr. J. F. Hughes, Assistant Conservator of Forests, for much help in the statistical examination of data and Mr. Peter Joachim, African Veterinary Assistant, for his conscientious and careful work on the herds used in the experiments.

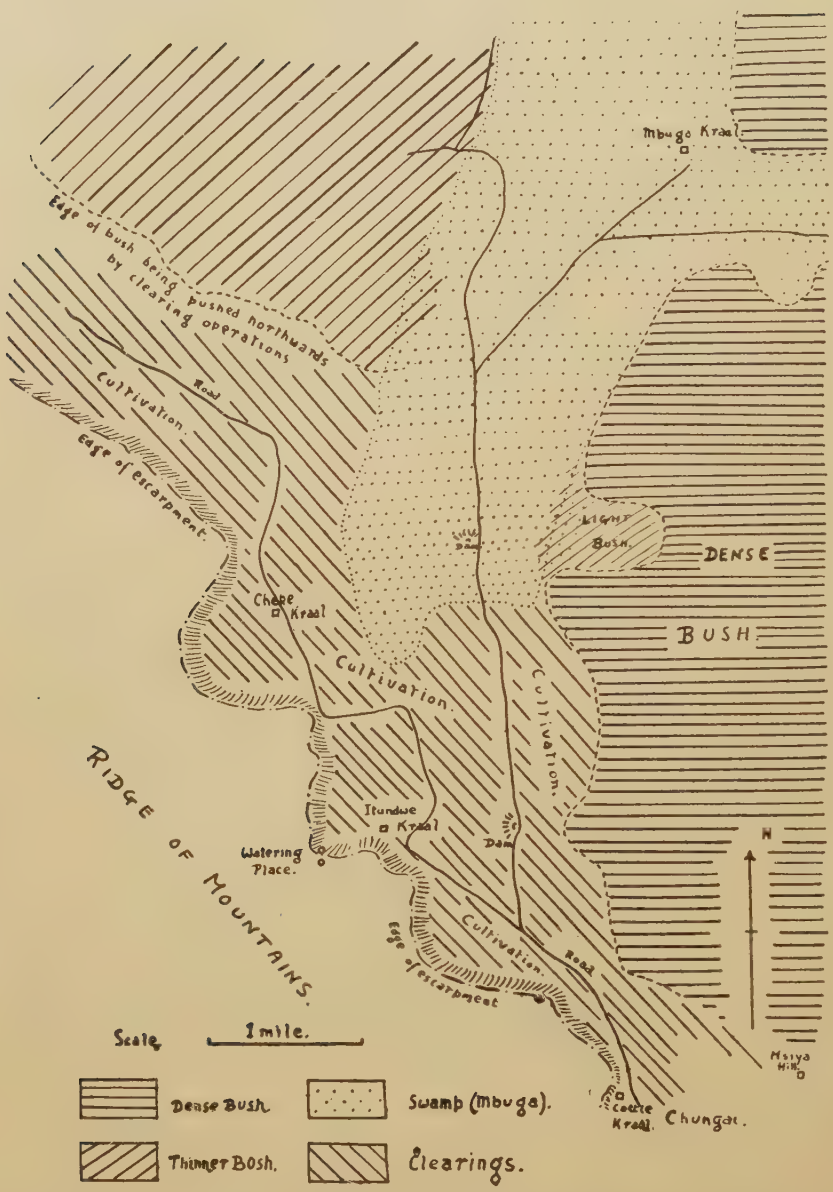
My thanks are also due to the Director of Veterinary Services for permission to publish this paper.

## REFERENCES

Hornby, H. E., Evans, S. A., and Wilde, J. K. H. (1943). *J. comp. Path.*, **53**, 269.

Mainland, D. (1938). *The Treatment of Clinical and Laboratory Data.* Edinburgh : Oliver and Boyd.

[*Received for publication May 30th,* 1947.]




SKETCH MAP OF THE AREA IN WHICH THE EXPERIMENT WAS (DRAWN FROM A MAP PREPARED BY [illegible]) CARRIED OUT.

# A STUDY OF THE RE-EXPOSURE TO *TR. FOETUS* OF ANIMALS ALREADY EXPOSED TO THE INFECTION AS VIRGIN HEIFERS, WITH SOME OBSERVATIONS ON THE LOCALIZATION OF ANTIBODY IN THE GENITAL TRACT

By

W. R. Kerr and Muriel Robertson,

*Veterinary Department, Ministry of Agriculture, Northern Ireland, and the Lister Institute, London*

## INTRODUCTION

Trichomoniasis of cattle has frequently been described by observers in the field as a self-limiting disease. This self-limiting character has two aspects. In the first place there is the spontaneous disappearance of the parasites from infected animals without permanent loss of breeding capacity (Laing, 1947) and secondly the acquisition of some degree of resistance to further exposure to infection, (Andrews and Miller, 1938). Nevertheless, outbreaks keep recurring in infected districts with disappointing frequency.

In 1944, the present authors carried out an experimental study of the infection of animals vaccinated parenterally with *Trichomonas foetus* and of a control group which had not been vaccinated. The method of infection used was the introduction of living *Tr. foetus* along with undiluted semen at artificial insemination. The results (Kerr and Robertson, 1946) showed that in the conditions of the experiments there was no protection afforded by a degree of vaccination sufficient to produce circulating antibody in the blood and its fixation in the skin.

The present communication deals mainly with the further exposure to infection with *Tr. foetus* of the animals used in the 1944 experiments. The immune body reactions have also been followed and some observations have been made upon the localization of antibody in the genital tract. The purpose of the enquiry at this stage was to see how animals whose reactions had been carefully observed on the first occasion would respond to further exposure.

## METHODS AND MATERIAL

The strains of *Tr. foetus* used were the "Belfast" strain isolated in 1939 in Northern Ireland and the "Manley" strain isolated from an outbreak in Cheshire. (Mahmoud, 1944). These two strains are serologically different from each other though the antigens overlap to some extent (Kerr and Robertson, 1945).

The infections were all carried out with living Trichomonas introduced along with the undiluted semen at insemination.

## EXPERIMENTAL RESULTS

The detailed history of the first exposure in the 1944 experi-

ments is given in the earlier paper (Kerr and Robertson, 1946); the animals were then virgin heifers.

The results of re-exposure to infections are stated in brief tabular form in Table I. The individual reactions are however of considerable interest and are given below.

Group 1. (D2, D4, and A9). Animals resisting the first exposure to infection in 1944, producing a calf at full term.

D2. (*not vaccinated*). This animal failed to take at first exposure in 1944 to "Belfast" strain. She was exposed to "Manley" strain on 3.9.45, 65 days after calving. She returned at three weeks, was re-infected with the same strain and returned again at 64 days. Trichomonads were not seen, but as the skin test had become positive 55 days after the exposure on 24.9.45 and remained positive for five months, it seems highly probable that the infection had taken. The blood titre did not rise significantly, but this does not imply that there was no circulating antibody in the blood stream.

This animal is recorded as a probable infection on 24.9.45. The exposure at the two consecutive inseminations may be considered as a second and third exposure or they may be taken together as constituting an effective second exposure (as in Table I.) On the day of the second return in the present series, the animal was inseminated and again exposed to "Manley" infection. The infection was resisted and the animal calved to term.

This animal can be considered to have failed to take at the very first exposure on 11.9.44 to the "Belfast" strain; to have taken with the second exposure on 24.9.45 ("Manley") and to have resisted the third exposure on 31.1.46 ("Manley").

D4. (vaccinated 1944, strain "Belfast"). This animal resisted the first exposure in 1944 and calved on 28.7.45. Five weeks before the re-exposure to infection the blood no longer showed a titre above the normal from the vaccination although the skin test was still positive. Seventy-one days after calving she was inseminated and exposed to infection with "Belfast" strain. Trichomonads were seen by direct examination on the 12th and on the 27th days, and the animal returned to season at 31 days. When inseminated and exposed to infection with "Manley" strain two months later (14.1.46) the infection failed to take and the animal calved to term on 20.10.46.

Thus there was a clear infection at the second exposure and a failure to infect at the third. We do not consider that the evidence is conclusive that the animal had a specific acquired immunity to *Tr. foetus* as the change of strain from "Belfast" to "Manley", which are serologically different, makes this unlikely. We are inclined to consider that it is an expression of the general resistance in cows to infection and this is borne out by the subsequent history.

This animal was vaccinated intramuscularly during the last months of the 1946 pregnancy with strain "Belfast." Eight weeks

TABLE I

RESULTS OF RE-EXPOSURE TO INFECTION

| *Animals resisting first exposure* (strain "Belfast") | *Animal which had subclinical infection at first exposure* | *Animals which had acute infection at first exposure* (strain "Belfast") | *Result of second exposure* | *Third and subsequent exposures* | *Remarks* |
|---|---|---|---|---|---|
| D 2 not vaccinated | — | — | Took at second exposure returned at 64 days. Strain "Manley." | Resisted third exposure. Strain "Manley." Calved to term. | Failed to take at first. Took at second exposure with different strain ("Manley"). |
| D 4 vaccinated strain "Belfast" | — | — | Took at second exposure. Trichomonads seen. Returned at 31 days. Strain "Belfast." | Resisted third exposure. "Manley" strain used. Calved to term. | — |
| A 9 not vaccinated | — | — | Took at second exposure. Strain "Manley" used. | — | — |
| — | A 18 not vaccinated. Low passive titre in early calfhood. Strain D 8 passage of "Belfast." | — | Took at second exposure. Trichomonads seen. Strain "Manley." | Took at third exposure. Strain "Manley" used but query if this is really a *new* infection. | The subclinical infection with D 8 lasted seven months but pregnancy not interrupted. Took when exposed to strain "Manley." |
| — | — | D 6 vaccinated strain "Belfast." Aborted 1944-45 experiments. | Resisted second exposure to strain "Belfast." Calved to term. | Took at third to fifth exposure to strain "Manley." No trichomonads found at *post-mortem* examination or cultivated. | — |
| — | — | D 7 vaccinated strain "Belfast." Aborted 1944–45 experiments. | Resisted second exposure to strain "Belfast." Calved to term. | — | — |
| — | — | D 8 vaccinated strain "Belfast." Aborted 1944–45 experiments. | Resisted second exposure to strain "Belfast." Calved to term. | — | — |

after calving she was exposed to 20 million organisms of strain "Manley" at insemination (16.12.46). Trichomonas were numerous from the 19th to the 23rd day and she returned to season on the 31st day. She was then exposed to 5 million Trichomonas. On the 16th day, trichomonads were numerous in the vagina and on the 21st day (6.2.47) she was again in season. It is not certain that this was a new infection as there is evidence in another animal (D6) in this series that Trichomonas even in cows can persist through the normal oestrus cycle. In this animal the resistance shown on 14.1.46 to strain "Manley" was no longer operative on 16.12.46.

This is a particularly interesting animal. She was not exposed again to infection, but as shown in a later part of the paper the infective period of 16.12.46 to 6.2.47 resulted in the localization of immune body in the uterus as demonstrated by the agar plate test with the living organisms (Pierce, 1947). (See Table III).

A9. (*not vaccinated*). This animal failed to take at the first exposure to strain "Belfast" at insemination on 2.1.45 and calved 25.9.45. The animal was left for one year and was then inseminated with 5 million of strain "Manley" introduced along with the sperm (30.9.46). In 17 days trichomonads were present in the vaginal discharge and fluctuated in numbers during the next three weeks but were particularly numerous from 14.11.46 to 18.11.46. A week later (56th day) the animal returned to season and was inseminated but was not re-exposed to infection; she returned again in 38 days. At *post-mortem* examination on 21.1.47, the uterus was normal in appearance and trichomonads were neither seen nor could they be cultivated. It should be noted here that the first exposure in 1945 was to strain "Belfast" while the second exposure in 1946 was to strain "Manley."

The three animals of this group all became infected at the second exposure and the result suggests that the failure to become infected on the first occasion was not the expression of an effective particular resistance to the organism, but represents, as it were, the natural resistance to the disease in the conditions of the experiment.

Group 2. An animal which had a subclinical infection as a result of the first exposure.

A18. (*not vaccinated*). This animal harboured Trichomonas of strain "Belfast" in the uterus for at least seven months after the first exposure but calved normally on 5.9.45.

A year later (21.9.46) this animal was exposed to 10 million strain "Manley" at insemination. The infection took and trichomonads were found by direct examination on the 16th day with return of oestrus on the 26th day (17.10.46). She was immediately re-exposed to infection, 2 million strain "Manley" trichomonads being introduced with the semen.

Trichomonads appeared again on the 22nd day in large numbers and the animal returned on the 38th day. Insemination without

infection was then carried out at once and there was a return of oestrus on the 41st day. At the *post-mortem* examination a fortnight later the uterus was normal and *Tr. foetus* was not found at examination nor on cultivation. It is again not certain that the exposure on 17.10.46 was necessarily responsible for the organisms appearing on the 22nd day after that date. The possibility of a persistent earlier infection (21.9.46) cannot be excluded.

There was no evidence that the low infection with "Belfast" strain in the first pregnancy had any effect at all on the response to the subsequent exposure. The interval of one year after the first subclinical infection and the fact that the strain used was "Manley," whereas the first exposure had been to "Belfast," must be taken into account in considering this animal.

Group 3. (D6, D7 and A2). Animals which had an acute infection with strain "Belfast" at the first exposure.

D6. (*vaccinated* 1944 with strain "Belfast"). This animal aborted at 16 weeks as a result of the infection produced by the first exposure to strain "Belfast" at insemination in 1944; a pyometra had developed before abortion. Two and a half months later when both the blood and the skin test were positive the animal was exposed at insemination to infection with strain "Belfast" (13.4.45). Trichomonads were never seen and she calved at full term (14.1.46). Previous to calving she had received a vaccinating dose of strain "Manley" (9.1.46). On 7.10.46, nine months after calving normally, this cow was exposed at insemination to infection with 5 million Trichomonas (strain "Manley"). Trichomonads were not seen and the animal returned in ten days. She was again inseminated and exposed to infection with 2 million "Manley" strain. She returned in 25 days (11.11.46) but trichomonads were not seen. The infection and insemination were again repeated for the third time since the normal calving and the animal returned again at 17 days. She was now inseminated without infection and returned after 53 days (21.1.47) : at *post-mortem* examination 24 hours later the uterus appeared normal. Trichomonads which had not been seen at direct examination were cultivated from the uterus.

Two interesting things are shown by the results in this animal : (1) that failure to conceive may be connected with low Trichomonas infections where the parasites are not sufficiently numerous to be observed by direct examination and (2) that trichomonads can persist in a cow for at least 67 days and through two oestrus cycles.

The Trichomonas cultivated on 21.1.47 must derive from one of the three exposures taking place between 7.10.46 and 11.11.46, most probably from the last exposure. Cows with a similar history to D6 during this last period are familiar to every veterinary officer who has had to deal with infected herds.

D7. (*vaccinated* 1944 with strain "Belfast"). This animal aborted at 14½ weeks (8.2.45) after the first exposure to strain "Belfast" in 1944 : pyometra developed before abortion. She was

treated by uterine irrigation with 1/400 iodine on 2.3.45. Three months after the abortion and two after the irrigation the animal was exposed to infection with " Belfast " strain at insemination. The blood titre was still above normal (14½/9) and the skin test was also positive.

The infection did not take, trichomonads were never seen and the animal calved at full term. The calf was fully developed but failed to suck. D7 was not again exposed to infection.

D8. (*vaccinated* 1944 with " Belfast " strain). This animal exposed to strain " Belfast " for the first time in 1944, aborted at 18 weeks, without pyometra. The date of the abortion was 20.2.45 and she was treated by uterine irrigation with iodine 1/400, 11 days later. She was inseminated and exposed to infection with strain " Belfast " on 6·6.45 (3½ months after the abortion), when both the blood titre and the skin reaction were positive. Trichomonads were not seen and the pregnancy developed normally, a healthy calf being produced on 18.3.46. Six months later (23.9.46) the animal was exposed at insemination to 10 million organisms of strain " Belfast." The animal returned to oestrus in 16 days with numerous trichomonads in the discharge.

D8 is a very clear case of an animal having an acute infection at the first exposure resisting the second exposure, but becoming infected at the third exposure. The one strain " Belfast " was used throughout.

To complete the evidence of re-infection it seemed interesting to include an animal where the organisms were transmitted by the bull.*

A2. (Virgin heifer). This animal was served 19.7.41 by the infected bull.

On the 45th day, *Tr. foetus* was observed in the vaginal secretion but not in great numbers. On the 49th day (6.9·41) the animal was in oestrus and was again served by the same bull. On the 18th day after this service trichomonads were numerous in the vaginal discharge and on 23.10.41 which was the 47th day since the second service and the 96th day since the first service, the skin test was positive although the level of circulating antibody had not risen appreciably above the normal in the serum test. The parasites were again numerous on the 62nd day and 19 days later the animal returned to service at 12 weeks.

This is probably an example of re-infection by the bull although it is not possible to be quite certain owing to the occurrence of cases

---

* Another animal of the 1944-45 series can be added to the acutely infected animals which resisted re-infection.

B17 not vaccinated. Had a passive titre in early calfhood acquired by way of the alimentary canal. This had disappeared by the 8th month of life. Infected when 18 months old in the 1944-45 experiments (Kerr and Robertson 1946) with strain "Manley". Took an acute infection and aborted at 13 weeks. Re-exposed to infection with strain "Manley" at insemination 3½ months after abortion. The infection failed to take and the cow calved normally.

of survival of Trichomonas described by Hammond and Bartlett (1945) in heifers and by the present writers here in the case of D6. The pronounced type of the second infection suggests however that it is a more effective re-infection rather than a carry over from the first service.

## LOCALIZATION OF IMMUNE-BODY

The experimental work carried out with these re-infected animals and some others with which we were working at the time included many tests of the vaginal mucin by Pierce's agar plate method and his discovery that in infected animals antibody against Trichomonas is found in the mucin from this site was confirmed (Pierce, 1947).

During this study of re-infection an examination of the uterus was made for the presence of immune-body.

The method entails the injection into the body of the uterus of a quantity varying between 50 and 100 ml. of sterile physiological saline and the withdrawal of some of this fluid without possible contact with the vaginal mucin. The fluid is found to contain uterine mucin in varying degrees of dilution and in some cases a few red blood cells.

The sterile catheter is passed in the usual way. The cervix is fixed by vulcellum forceps in preference to the rectal manipulation, and sterile saline solution is then injected by means of a syringe of a capacity of 100 ml. Having injected the solution the syringe is detached and filled with air which is forced through the tube and catheter to empty out the residue of saline solution. The piston is then drawn back slowly whilst the point of the catheter is gently moved backwards and forwards. The syringe is detached and the contents injected into a sterile tube. In many cases the fluid will be found to syphon off quite freely. The point of the catheter must not be withdrawn into the cervix or vagina as mucin from these surfaces would be drawn into the catheter whilst some degree of suction is in the tube. By the use of this technique the mixing of the uterine wash with the vaginal mucin is prevented.

It should be noted that a few red blood cells were present in both positive and negative samples.

### *Localization of antibody in an animal examined after infection with living organisms*

The animal D4 of the re-infection series already described was examined by the above method 63 days after the last positive observation of Trichomonas by direct examination (1.2.47). There had intervened three returns of oestrus, the first on 6.2.47, the second on 1.3.47 and the third on 22.3.47.

The cow had been artificially inseminated on the first two dates but not on the third. The sample was therefore taken 13 days after the oestrus of 22.3.47 and ten days before the next oestrus on 14.4.47. The cow is now in calf to insemination carried out artificially on this date. The importance of this last observation is that

the presence of the antibody in the uterus has not prevented conception.

The material withdrawn from the uterus was set up in the agar plates as described by Pierce (1947). The test also included a sample of mucin taken from the vagina three days after the uterine sample. (See Table II). The dilution of the uterine material cannot be accurately ascertained but the amount of saline introduced to obtain the " uterine wash " was always 50 to 100 c.c.

On this occasion the agglutination test gave + + in a dilution of 1 : 80 of the " uterine wash " and + + + in 1 : 80 dilution of the vaginal mucin. This observation in a cow with the history of D4 is of obvious importance and opens up a much more detailed and realistic approach to the study of acquired resistance to re-infection with *Tr. foetus*. In this animal the presence of the immune-body in the uterus was the result of infection with the live organism.

It should be noted that a large proportion of uterine examinations of animals exposed to infection have been negative as well as all the samples from normal animals. The negative controls constitute a satisfactory basis for this part of the investigation.

*Localization of antibody in animals where the antigen had been instilled into the uterus*

In the acute disease the organisms are, for at least some part of the time, in contact with the epithelium lining the uterus and the antigen is absorbed from this site. (Kerr and Robertson, 1943). It seemed worth while to investigate the state of the uterine secretion in animals in which the antigen had been artificially introduced as a guide to what might be taking place in the case of an infection with the living organism.

The antigen was introduced into the uterus by means of a catheter and the animal (D14) was prepared by several infusions. The detailed history is as follows :—

D14 was a normal calved cow which had been under observation at the laboratory of the Ministry of Agriculture, Northern Ireland, but had not been used. Blood titre and skin test were both negative.

Vaccination was carried out with dried powder " Belfast " antigen, ground up in saline. This antigen consisted of the crude freeze-dried Trichomonas organisms grown in Brownlee's medium (tryptic digest of horse muscle broth with 2 per cent. glucose and 7 per cent. bovine or horse serum added). The organisms were thoroughly washed in saline before they were dried. The total quantity was 0·5 g. given in three doses between 17.10.46 and 6.12.46. The blood test became positive before the end of the vaccination on 12.11.46 (index 17 : 9) and the skin test gave a reaction (7 to 11) on 29.12.46. Thirty-six days later 0·25 g. of crude dried " Belfast " antigen in 50 c.c. of saline was instilled into the uterus. As there was no reaction, it may be concluded that the uterus had not been sensitized by the parenteral vaccination. The

TABLE II

AGGLUTINATION OF LIVING TRICHOMONAS IN AGAR PLATE TESTS MADE ON SAME DAY

| *No. of animal and date of sample* | *Strain used for suspension* | *Nature of substance incorporated in plates* | *Dilutions* | | | | |
|---|---|---|---|---|---|---|---|
| | | | 1 : 10 | 1 : 20 | 1 : 40 | 1 : 80 | *Saline* |
| D 4 4.4.47 ... ... ... | " Manley " | Uterine wash | ++++ | +++ | ++ | ++ | 0 |
| D 4 7.4.47 ... ... ... | " Manley " | Vaginal mucin | ++++(+) | ++++ | ++++ | +++(+) | 0 |
| D 16 3.3.47 ... ... | " Manley " | Vaginal mucin | 0 | 0 | 0 | 0 | 0 |
| Known positive " Manley " serum | " Manley " | Known positive " Manley " bovine serum | ++++(+) | ++++ | +++(+) | +++ | 0 |
| Known normal bovine serum | " Manley " | Known normal bovine serum | ++ | + | (+) | (+) | 0 |

D 4 is an animal recovered from infection. *Trichomonas* last seen 63 days before uterine sample taken.

instillation was repeated seven days later, also without reaction. A larger dose of 0·5 g., instilled 17 days later, produced a moderate, but quite definite, local reaction so that two hours after the instillation the uterus had become hard and rigid and had increased very markedly in size. At four hours the effect had disappeared. It therefore could be concluded that the previous instillations had sensitized the uterus. Eight days later uterine and vaginal samples were taken separately from this animal by the technique already described. Table III gives an account of the tests of D14 and of two other animals.

The uterine wash and the vaginal mucin of D14 both contained demonstrable antibody in considerable amounts. Two other animals which had also had antigen instilled into the uterus were tested on the same day. D13 showed antibody in the uterine wash but not in the vaginal mucin and D18 showed no antibody in the uterine sample but had a positive titre in the vaginal mucin.

It is not intended to go further into the details of the conditions in which the antibody is localized in this site in this communicaton, but the data suggest that it is connected with the sensitization of the uterus.

These results are of obvious importance in the whole matter of resistance and re-infection, but a great deal more observation of natural infections and experimental work is required to appreciate the role of the uterine immune body in the actual protection against infection. Much more investigation is also needed of the exact conditions under which the antibody is formed and of the mechanism of its fluctuation and disappearance. Its relation to the oestrus cycle must also be studied. The significance of the original observations on the relation of the localization of the antibody to the immune body circulating in the blood stream is at present obscure but can be approached experimentally.

## DISCUSSION

In reviewing the data of re-infection it is obvious that the positive nature of the evidence of the break-down of immunity is much more convincing than that brought forward in support of the establishment of some degree of protection by a previous acute infection. This is partly due to the fact that we cannot claim 100 per cent. success in any form of infection whether by the bull or by artificial introduction of the organisms at insemination.

Nevertheless the experiments recorded here in which three acute infections appear to have protected three animals (D6, D7 and D8) from a relatively rapidly ensuing re-exposure, do lend support to the considerable degree of self-limitation observed in field outbreaks. D2 might be added to this group as the "Manley" infection of 24.9.45 might be considered to have protected her against the exposure to the same strain on 3.1.46. The occurrence of immune body localized in the uterus gives actuality to this claim and its

TABLE III

AGGLUTINATION OF LIVING TRICHOMONAS IN AGAR PLATE TESTS MADE ON SAME DAY
Three animals artificially sensitised by instillation of antigen

| *No. of animal and date of sample* | *Strain used for suspension* | *Dilution of uterine wash* | | | | | *Dilution of vaginal mucin* | | | | | *Index of blood test* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 : 10 | 1 : 20 | 1 : 40 | 1 : 80 | *Saline* | 1 : 10 | 1 : 20 | 1 : 40 | 1 : 80 | *Saline* | |
| D 14 12.3.47 | "Belfast" | ++++ | ++++(+) | +++(+) | +++ | 0 | ++++ | +++(+) | +++(+) | +++(+) | 0 | 18/9 |
| D 13 12.3.47 | "Belfast" | ++(+) | +++ | +++ | ++ | 0 | 0 | 0 | 0 | 0 | 0 | 17/9 |
| D 18 12.3.47 | "Manley" | 0 | 0 | 0 | 0 | 0 | +++(+) | +++ | ++(+) | ++ | 0 | $12\frac{1}{2}$/9 |

further investigation will bring some precision into this aspect of protection.

Immune body in the vaginal mucin (Pierce, 1947) probably accounts for the gradual disappearance of *Tr. foetus* from those animals in which the organism persists after the return of oestrus and is in sharp contrast to the rapid clearing out of the organisms after abortion in acute types of the disease (Kerr and Robertson, 1946). The experimental results recorded here show that its role in the actual prevention of infection does not seem to be very great. In A2, for example, the bull seemed to be able to infect the animal in spite of the probable presence of antibody in the vaginal mucin : here again further work will clarify the position.

A matter of interest from the breeders point of view is the case of D6 where relatively small numbers of Trichomonas introduced with the sperm appeared to prevent conception without the organism being sufficiently numerous to be observed by direct examination. The survival of trichomonads in this animal for at least 67 days through two oestrus cycles should be noted. The animal was a cow which had had a previous abortion in the vaccination experiments of 1944-45, and a subsequent normal pregnancy. This brings her out of the heifer class where trichomonads may be found to persist at times through as many as four oestrus cycles (Hammond and Bartlett, 1945).

In considering the difficulty in getting animals in an infected herd into calf the fact that at *post-mortem* examination the uterus was normal in three animals which failed to conceive, is of interest as it suggests that persisting trichomonads may more often account for the sterility than actual damage to the uterus. This might also account for the degree of success obtained by iodine irrigation in re-establishing breeding.

The main conclusion to be drawn is that, while some degree of protection appears to be established by a previous acute infection, its duration is, relatively speaking, short and it does not seem to persist beyond a normal pregnancy.

## SUMMARY

Animals whose reactions to exposure to infection with *Trichomonas foetus* at insemination had been observed in detail in 1944-45 were re-exposed to infection at times varying from six and a half to 21 months after the first infection.

Three animals which had failed to take on the first occasion all developed infections on the second occasion.

Three animals which had had acute infections at the first exposure all resisted the second exposure and calved to term, but this did not prevent infection on further exposure after the normal pregnancy.

An examination of the uterus by a technique described in the text showed that in one infected animal and in several animals

artificially sensitised to uterine instillation there was a localisation of antibody in the uterine secretion.

The vaginal mucin of infected and sensitized animals was examined and the localization of antibody in this site, first described by Pierce (1947), was confirmed.

REFERENCES

Andrews, J., and Miller, F. W. (1938). *Amer. J. Hyg.*, **27**, 235.
Brownlee, G. (1943). Personal communictaion of medium for growth in bulk of *Trichomonas foetus*.
Hammond, D. M., and Bartlett, D. E. (1945). *Amer. J. vet. Res.*, **6**, 84.
Kerr, W R., and Robertson, M. (1943). *J. comp. Path.*, **53**, 280 ; (1945). *Vet. Rec.*, **57**, 221 ; (1946). *J. comp. Path.*, **56**, 101.
Laing, J. A., and Day, P. T. (1947). *Vet. Rec.*, **59**, 265.
Mahmoud, A. H. (1944). *Ann. trop. Med. Parasit.*, **38**, 291.
Pierce, A. E. (1947). *J. comp. Path.*, **57**, 84.

[*Received for publication June 4th*, 1947]

# IRON METABOLISM IN PIGLET ANAEMIA

By

J. A. J. Venn, R. A. McCance and E. M. Widdowson

*Institute of Animal Pathology and the Department of Experimental Medicine, Cambridge*

## INTRODUCTION

Piglet anaemia was first described in this country by McGowan and Crichton (1923; 1924, a, b). They observed it in the litters of sows which had been placed under cover in concrete pens a fortnight before farrowing. It has now been established that piglets reared under these or similar conditions are very prone to develop an anaemia during the second and third weeks of life and that the anaemia can be prevented or cured by administering iron salts to the piglets. Small amounts of copper in addition to the iron are thought by some to be beneficial (Elvehjem and Hart, 1932; Kernkamp, 1935; Moe, Craft and Thompson, 1935; Schültze, Elvehjem and Hart, 1936), but the anaemia is primarily one of iron deficiency. Dosing the piglets is a slow and tiresome task, and much effort has been directed towards finding some alternative. McGowan and Crichton originally claimed that the anaemia could be prevented by adding ferric oxide to the sow's food. This has not generally been confirmed (Doyle, Mathews and Whiting, 1928; Hart, Elvehjem, Steenbock, Kennerer, Bohstedt and Fargo, 1929–30), nor has a claim of Doyle (1932) that the piglets did not become anaemic if their mothers had been kept on pasture right up to the time of farrowing. The apparent efficacy of marrow stem kale and iron fed to pregnant sows (Venn, 1944) was not confirmed by further work of the author, a finding that was supported by Braude and Foot (1946). It is, however, well established that if piglets are reared on pasture from birth, or have access to soil or sods in concrete pens, they do not become anaemic (Hamilton, Hunt, Mitchell and Carroll, 1930; Schofield, 1931; Fulton, 1932; Kernkamp, 1935, 1941; Moe, Craft and Thompson, 1935; Baskett and Lamont, 1936; Fargo, 1936). It has further been shown that spontaneous recovery commences at weaning time provided the anaemia has not been so severe as to produce permanent stigmata.

Lintzel, Rechenberger and Schairer (1944) have reported the results obtained by an analytical examination of two piglets at birth and one aged 12 days. They found that newborn piglets weighing about 1,200 g. contained 36 mg. of iron, of which 16 mg. were in the form of haemoglobin and 8 mg. were in the liver. The piglet analysed on the 12th day of life contained 77 mg. of iron, 47 mg. circulating as haemoglobin and 0·5 mg. in the liver. Lintzel *et al.* assumed that the increment of iron had come from the milk and the soil, and pointed out that the liver contained enough iron at birth to be of some importance as a store. Apart from this, little work appears to have been done on the metabolism of iron in piglets, and since the

absorption and disposal of this element seems to be of the greatest importance to the well-being of these animals, it occurred to us that a study of it might be well repaid. Suckling anaemia, moreover, is not confined to piglets, and is not uncommon in the human infant, particularly in those who are born before full term. It was thought, therefore, that the results obtained might have implications outside the pig rearing industry.

## METHODS

The animals were kept at the Institute of Animal Pathology, Cambridge, in concrete pens with a covered sty attached to each. The sows were Large White or first-cross Wessex/Large White, mated to a Wessex boar. The sow's food consisted of pollards, 60 per cent.; beanmeal, 30 per cent.; and fishmeal, 10 per cent. The animals were fed twice a day and they were given 7 lb. daily during pregnancy and 10 lb. daily during lactation. The sties were kept very clean and faeces were swept out at frequent intervals, particularly if iron was being administered to the lactating mother or to some, but not to all, of the piglets. Weaning was commenced when the piglets were three weeks old and the sow was removed at eight weeks. One litter was reared on grass. Crude ferric oxide was used to supplement the sow's ration in some instances. It was given from the fourth week of pregnancy onwards, in doses of, roughly, 12 and 16 g. per day during pregnancy and lactation respectively. Ferrous sulphate was used for the piglets in a dose of roughly 5 mg. Fe per pound of bodyweight per day. The sow's milk was obtained by expression from the cleaned nipples into clean glass tubes. The amount of milk taken by the piglets was determined by "test-weighing." The piglets were removed from the sow and taken back to feed at two-hourly intervals. They were placed in bags and weighed immediately before and after feeding.

The piglets were brought to the Department of Experimental Medicine on the morning of the day they were to be killed and their blood volume was determined without an anaesthetic by means of Evans blue. A preliminary sample of blood was taken from the ear, the internal jugular vein or the heart, and the plasma separated. A mixture of ammonium and potassium oxalates was used as the anticoagulant. Then a measured amount, usually 5 c.c., of a solution of Evans blue in 0·9 per cent. saline was injected into one or other of these places. The concentration of the solution of Evans blue was such that 10 mg./kg. bodyweight were administered. It was hoped that the use of high concentrations of Evans blue would minimise the errors caused by the activity of the reticulo-endothelial system (Courtice, 1943) as well as making the determination easier to carry out. A second sample of blood was taken from the heart after six minutes. Part of this was used for haematocrit determinations, some was ashed for the determination of its iron content, and the remainder was centrifuged and the plasma separated. After

suitable dilution with 0·9 per cent. saline, the coloured plasma was matched against the control serum to which a measured amount of the diluted dye had been added. The piglets were killed by administering an overdose of chloroform or by injecting air into the heart. After death the animals were dissected on a large enamel tray so that no blood was lost, the intestines were washed out, particularly if the animals had been given iron, and portions of the liver and spleen were taken for the determination of inorganic and total iron. The remainder of the carcase was cut into pieces and placed in vessels containing moderately strong HCl. The trays were washed and the washings added to these vessels. After the bones and organs had softened at room temperature they were transferred to large beakers, heated in a water bath for some days and finally over a flame until nearly all the solid matter had all gone into solution. By appropriate manipulation of the volumes and sampling, a solution was finally obtained representing whole animals, and suitable aliquots of this were taken for analysis. Fat presented no difficulties when the piglets were newly born but, if in older animals a thick enough layer collected on the surface of the beakers, the hydrolysate was cooled to 0° C., the fat removed and sampled separately after making up to a suitable volume with alcohol. Measured amounts of the acid hydrolysate and of the whole blood were evaporated to dryness in silica crucibles and then ashed at 450° C. overnight. The ash was moistened with conc. HCl and gently heated to dryness on a sand bath. Subsequent treatment was exactly as described by McCance, Widdowson and Shackleton (1936).

Total iron was determined by the thioglycollic acid method (McCance, Widdowson and Shackleton, 1936) and the inorganic iron in the internal organs by Hill's (1930) method, as modified by Shackleton and McCance (1936). Haemoglobin in blood was determined by Sahli's method in which 100 per cent. is equivalent to 14 g. haemoglobin/100 c.c., and haematocrit by spinning the blood for 45 minutes in thick walled glass tubing of 2 mm. internal diameter at 3,500 r.p.m.

## RESULTS

### *Variations between litters, and the effect of size on the composition of litter mates at birth*

The quantity of iron in the bodies of newborn pigs varied from 1·92 to 3·48 mg./100 g., and the mothers of both these piglets had been given iron throughout pregnancy. The variations within any one litter, however, were much smaller, and it is remarkable how constant the percentage composition of litter mates may be in spite of great variations in their size. This is shown by the figures for litters Q and T in Table I. The figures for litter S, however, appear to show that size may sometimes make a small difference, so that its effects cannot be entirely excluded on the basis of the available data.

TABLE I

THE EFFECT OF SIZE ON THE COMPOSITION OF LITTERMATES AT BIRTH

| *Litter Q* | | *Litter S* | | *Litter T* | |
|---|---|---|---|---|---|
| *Body weight* g. | *Iron* mg./100 g. | *Body weight* g. | *Iron* mg./100 g. | *Body weight* g. | *Iron* mg./100 g. |
| 1,236 | 3·28 | 1,331 | 3·48 | 1,448 | 2·62 |
| 864 | 3·22 | 1,178 | 3·02 | 1,155 | 2·74 |
| 472 | 3·13 | 997 | 2·86 | 1,035 | 2·66 |
| | | 890 | 2·63 | | |

*The effect of administering iron to the sow during pregnancy on the composition of the piglets at birth*

This is a fundamental question, not only in the prevention and treatment of piglet anaemia, but also in the whole realm of pre-natal nutrition, and an attempt has been made to solve it by analysing some of the progeny of sows which had, or had not, been given iron during pregnancy. In the first instance, the results obtained on newborn piglets from six sows which had been given iron during pregnancy were averaged and compared with the average findings for newborn pigs from four sows which had received no supplementary iron. The results are set out in Table II, from which it

TABLE II

THE EFFECT OF FEEDING IRON TO THE SOW DURING PREGNANCY ON THE COMPOSITION OF NEWBORN PIGS

| | *Sow given Fe during pregnancy* | *Sow not given Fe during pregnancy* |
|---|---|---|
| Number of litters studied ... | 6 | 4 |
| Average number in litter ... | 10 | 12 |
| Average weight of individual piglets produced g. ... ... | 1,548 | 1,383 |
| Average weight of living matter in each litter g. ... ... | 15,480 | 16,650 |
| Average Fe in whole body mg. per 100 g. ... ... ... | 2·93 | 2·92 |
| Average inorganic Fe in liver and spleen mg. per 100 g. ... ... | 9·7 | 9·5 |
| Average concentration of Fe in blood mg. per 100 g. ... ... | 30·0 | 31·6 |

will be seen that there is no evidence that the administration of iron raised the percentage of this element in the piglets or the total quantity transmitted to the progeny through the placenta. This conclusion may be supported by data for the haemoglobin levels at birth. The results embracing a larger number of animals and carried out by J.A.J.V. alone, are given in Table III ; they do not suggest

TABLE III

THE PERCENTAGE OF HAEMOGLOBIN IN THE BLOOD OF PIGLETS AGED 0 TO 2 DAYS

| *Medication of sow during pregnancy* | *Numbers of litters studied* | *Numbers of animals tested* | *Haemoglobin g. per 100 c.c.* *average* | *range* |
|---|---|---|---|---|
| None ... ... | 9 | 46 | 12·3 | 10·1–14·7 |
| Iron as crude ferric oxide ... ... | 11 | 56 | 11·9 | 9·0–16·8 |

that the piglets derived any benefit from the iron which had been administered to their mothers. The problem, however, seemed such an important one that it was decided to study it in another way. Four sows and their progeny from two separate farrowings were used for this purpose. In the first season, two of the sows were given supplementary iron and two were not, and in the second the procedure was reversed, so that the effect of iron on the offspring of each individual sow could be assessed. A male and a female piglet from each of the eight litters was analysed, and the results are shown in Table IV. It so happened that each sow produced a

TABLE IV

THE EFFECT OF FEEDING IRON TO FOUR SOWS DURING PREGNANCY ON THE IRON CONTENT OF THEIR PROGENY

| | *Fe during pregnancy* | | *No Fe during pregnancy* | |
|---|---|---|---|---|
| Average number in litter ... ... ... | 13 | | 9 | |
| Average weight of individual piglets produced g. ... ... ... ... ... | 1,151 | | 1,512 | |
| Average weight of living matter in each litter g. ... ... ... ... ... | 14,390 | | 12 625 | |
| | *Male* | *Female* | *Male* | *Female* |
| Average Fe in whole body mg. per 100 g. ... ... ... ... | 3·33 | 3·37 | 3·08 | 2·98 |
| Average Fe in 1 piglet mg. ... ... | 38·4 | 38·8 | 46·6 | 45·2 |
| Average Fe in whole litter mg. ... | 482 | | 384 | |
| Average Hb. g. per 100 g. ... ... | 12·2 | 11·1 | 12·1 | 11·8 |

larger litter when she was given iron and the weight of the individual piglets was smaller. The iron supplement cannot have been responsible for it was not started until the fourth week of pregnancy. If the analytical data are inspected and compared, it will be seen that the administration of iron to the sows during pregnancy appears to have made them produce piglets with a rather higher concentration of iron in their bodies. The average amount of iron in a single piglet, however, was greater when the sows were not fed

iron because the individual piglets were considerably heavier. When the results are expressed as the total amount of iron in the whole litters, the beneficial effects of the iron supplement again become apparent, not only because the piglets contained more iron per 100 g. of bodyweight, but also because the litters were heavier.

The haemoglobin figures do not seem to run parallel with the concentration of iron in the bodies. This may be because the former estimations were made on all the piglets of each litter and an average of the results on all the piglets was taken, while the latter figures were derived from the results of two piglets only from each litter. Nevertheless, even if the administration of iron to the sows was the cause of the raised concentration of iron found in some of the piglets, the effects were so small that they would have done very little to prevent the development of the suckling anaemia. (See below.)

*The development and spontaneous disappearance of anaemia*

Litter mates were analysed at birth, three weeks and eight weeks of age, and the averaged results are set out in Table V. The findings

TABLE V

DISTRIBUTION OF IRON IN THE BODIES OF PIGLETS WHO HAVE RECEIVED NO SUPPLEMENTARY IRON

(All average results are calculated to the mean weight of the litter)

| | *Birth* | | *Three weeks* | | *Eight weeks* | |
|---|---|---|---|---|---|---|
| | *Average* | *Range* | *Average* | *Range* | *Average* | *Range* |
| Number of piglets analysed | 5 | | 5 | | 3 | |
| Weight g. ... ... ... | 1,529 | 1,223–1,920 | 6,510 | 5,253–8,250 | 11,872 | 9,412–14,000 |
| Total Fe in body mg. ... | 47·2 | 36·5–59·3 | 125·0 | 82·5–196·0 | 494 | 208–876 |
| Fe in body mg. per 100 g. | 3·09 | 2·62–3·48 | 1·92 | 1·40–2·37 | 4·15 | 2·20–6.25 |
| Total Fe in blood mg. ... | 42·2 | 27·9–57·0 | 82·5 | 48·3–131·0 | 384 | 141–540 |
| Fe in blood mg. per 100 g. | 28·3 | 22·9–33·8 | 14·1 | 13·0–16·3 | 32·9 | 20·1–35·0 |
| Inorganic Fe in whole liver and spleen mg. ... ... | 4·6 | 4·2–4·9 | 1·8 | 1·0–3·9 | 23·0 | 18·6–31·2 |
| Inorganic Fe in liver and spleen mg. per 100 g. ... | 10·40 | 7·7–12·1 | 0·76 | 0·38–1·24 | 5·58 | 4·50–6·60 |
| Fe in remainder of body mg. ... ... ... | 0·40 | | 40·7 | | 87·0 | |

require some comment. In the first place, it is necessary to consider the rate of growth of young piglets. Even the anaemic ones were four times their birth weight at the end of three weeks and eight times at the end of eight weeks. To avoid any reduction in the percentage of iron in their bodies, this growth-rate would require the deposition of about 7 mg. of iron per piglet per day for the first three weeks of life, and since milk was the only nourishment available, it is evident that the supply fell short of the demand. It will be observed, nevertheless, that the total iron in the body rose from 47·2 to 125 mg., so that each of the piglets obtained about 3·5 mg.

of iron per day. Had they not done so their anaemia would have been much worse, and would probably have proved fatal. As the anaemia developed, the percentage of iron in the body as a whole naturally fell, but the total amount of iron circulating in the blood rose from 42·2 to 82·5 mg. The liver lost about half its iron, and the percentage fell to a very low level. It is, however, important to realise that the amount of iron in the liver was only a very small fraction of the total iron in the body, even at birth, and that the capacity of the liver to act as an iron "store" was quite trifling. Thus the liver only contributed about 2·8 mg. of iron to the body as a whole during the development of the anaemia, and during this time the total iron in the body increased by some 78 mg. The quantity of iron in parts of the body other than the liver and the blood, underwent interesting changes. This estimation, however, is less exact than the others because it depends essentially on the differences between the total iron in the body and the iron in the blood, and the latter in turn hinges upon the determination of the blood volume, which cannot as yet be made with the precision of a chemical assay. The changes, however, were gross, and it would appear that the percentage of iron in the tissues rose rapidly in the first three weeks in spite of the development of the anaemia, and that the percentage continued to increase more slowly afterwards. It would appear, therefore, that the iron entering the body was shared between the tissues and the blood. Lintzel *et al.* (1944) reported a higher proportion of the total body iron in the tissues at birth than than has been found in the present experiments. Although the results now reported may be too low, it may be noted that Lintzel *et al.* perfused the body to remove the haemoglobin and estimated it in the perfusate and washings. Any haemoglobin remaining in the organs would, therefore, have increased fictitiously the so-called "tissue" iron. It is interesting, however, that with growth the German authors should also have found a great increase in the tissue iron/total body iron ratio.

Between three and eight weeks, when the piglets began to eat food other than milk, the total amount of iron in the bodies increased more rapidly than the weights, so that the percentage in the body rose. The concentration in the blood rose from 14·1 to 32·9 mg./100 c.c. The latter figure is higher than it had been at birth so that the blood was no longer deficient in iron, but the percentage in the liver had not reached the birth level by the eighth week.

### *The effect of administering iron to piglets during suckling on the composition of the body at three weeks*

The administration of iron had a profound effect on the well-being of the piglets and the results are given in Table VI. The analytical data are based upon six animals, but the changes in weight have been confirmed on many more. It will be noted that the piglets receiving iron put on more weight and, as might have been

expected, the amounts and concentrations of iron in the blood and livers of the piglets when they were three weeks old were greatly increased. There was also a smaller increase in the amount of iron in the rest of the body. It will be recalled that the percentage of iron in the rest of the body rose during the first three weeks of life while the animals were developing anaemia, and it may be that this fraction of the body is accorded a priority over the blood-forming organs when the supplies of iron are insufficient. The results presented in Table VI suggest that its demands are relatively well

TABLE VI

THE EFFECT OF FEEDING IRON TO PIGLETS DURING SUCKLING ON THE COMPOSITION OF THE BODY AT THREE WEEKS

| | *Piglets fed Fe* | | *Littermates not fed Fe* | |
|---|---|---|---|---|
| | *Average* | *Range* | *Average* | *Range* |
| Number of piglets analysed | 3 | | 3 | |
| Weight at 3 weeks g. | 7,817 | 6,400–9,600 | 6,927 | 5,500–9,500 |
| Increase in weight per 100 g. birth weight ... ... | 492 | | 420 | |
| Total Fe in body mg. ... | 286 | 188–350 | 108 | 74·5–153·0 |
| Fe in body mg. per 100 g. ... | 3·66 | 2·93–4·42 | 1·56 | 1·29–1·79 |
| Total Fe in blood mg. ... | 212 | 143–298 | 74·3 | 59·4–95·0 |
| Fe in blood mg. per 100 g. | 31·5 | 24·6–40·0 | 15·0 | 14·5–15·9 |
| Inorganic Fe in whole liver mg. ... ... ... | 22·5 | 9·6–30·4 | 3·8 | 2·5–5·6 |
| Inorganic Fe in liver mg. per per 100 g. ... ... ... | 9·5 | 5·0–14·2 | 1·72 | 1·6–1·8 |
| Fe in remainder of body mg. | 51·0 | | 29·9 | |

satisfied even when the general deficiency is great, and that it asks for and takes little more even in the midst of plenty. This is supported by a few analyses carried out on the individual organs by the more direct method of estimating the amount of inorganic iron in them. Thus the hearts of moderately anaemic and of the non-anaemic pigs each contained 2·2 mg./100 g. of fresh weight. The heart of an extremely anaemic pig, however, contained only 0·4 mg. per 100 g.

The way in which the administration of iron increased the weight of the piglets has been analysed by test weighing the piglets before and after suckling. Care was taken that the treated animals were well distributed between the front and rear teats. From the results in Table VII, it will be seen that the piglets that had been given iron took more milk, and this seems to be an adequate reason for their greater rate of growth. A somewhat similar observation was made by Houk, Thomas and Sherman (1946), who found that rats on a low iron intake ate less food and grew less rapidly than others whose diets contained more iron. These findings, however, do not exclude the possibility that the iron also increased the efficiency with which the animals dealt with their food.

Table VII

Amounts of Milk Consumed by Piglets Over Six Successive 2-Hourly Feeds

| *Litter* | *Age days* | *Number of piglets in each group* | *Average milk consumption per feed g.* | |
|---|---|---|---|---|
| | | | *Piglets fed Fe.* | *Littermates not fed Fe.* |
| 1 | 6 | 4 | 38·5 | 35·0 |
| 1 | 14 | 4 | 69 | 50 |
| 1 | 21 | 4 | 62 | 50 |
| 2 | 15 | 5 | 71 | 57 |

### *The beneficial effects of rearing the piglets with free access to grass and soil*

It has been known for a long time that piglets do not become anaemic if they are reared on pasture or have access to grass sods. This has been generally accepted as due to the iron in the soil, but it has never been specifically demonstrated. An analysis of the body of a three-week-old piglet which had been reared with free access to soil was, therefore, carried out. The mother received no iron medication. The results are given in Table VIII, and data derived from piglets reared in well-cleaned concrete pens have been added for comparison. The table also contains information regarding the composition of a piglet which had been reared in a concrete pen cleaned out once a day only, and to whose mother iron was administered during lactation. It will be seen that the piglets reared on cleaned concrete were very anaemic, and that the offspring of the

Table VIII

The Effect of Various Treatments during Suckling on the Composition of Piglets at Three Weeks

| | *No treatment to sow or piglets during suckling* | *Fe fed to sow during lactation Sty cleaned out once a day* | *No treatment to sow, but piglets reared outside with access to soil* |
|---|---|---|---|
| Number of piglets analysed | 5 | 1 | 1 |
| Average weight g. ... ... | 6,510 | 7,710 | 7,250 |
| Increase in weight per 100 g. birth weight ... ... | 327 | 418 | 328 |
| Total Fe in body mg. ... | 111 | 133 | 417 |
| Fe in body mg. per 100 g. ... | 1·70 | 1·72 | 5·76 |
| Total Fe in blood mg. ... | 82·5 | 108 | 300 |
| Fe in blood mg. per 100 g. | 14·1 | 16·3 | 35 |
| Inorganic Fe in whole liver mg. ... ... ... | 1·8 | 5·6 | 8·2 |
| Inorganic Fe in liver mg. per 100 g. ... ... ... | 0·76 | 2·20 | 3·80 |
| Fe in remainder of body mg. | 26·7 | 19·9 | 108·8 |

sow which was being given iron during lactation was little better. The slight improvement was no doubt due to the small amounts of iron picked up by the piglet in the pen, which was soiled with the sow's faeces by end of the day. In contrast, the piglet reared outside was well off for iron and its body contained more of this element than the bodies of those which had been given iron medicinally. (See Table VI.) Even the iron in the extra-hepatic tissues had been greatly raised. The reason for this abundance of iron was the soil eaten by the piglet. An analysis of washings from the gut yielded 513 mg. of iron. This was almost seven times the dose which the piglets shown in Table VI were being given per day, and indicated that the soil must have been exceedingly rich in iron. The amounts of iron in samples of 100 g. taken from the rearing paddocks and adjoining parts of the laboratory grounds were as follows: Lawn (ungrazed), 1·45 g.; cultivated land (cabbage) on which pigs had grazed the previous year, 1·32 g.; adjoining land uncultivated and ungrazed, 1·57 g.; and similar land on which pigs are now grazing, 1·32 g. Even so, the 513 mg. of iron found in this piglet's intestines cannot have come from less than 40 g. of soil. All soils will not be found to contain over 1 per cent. of iron, but these results show how much soil a piglet may ingest, and why the free access to soil prevents the development of any signs of anaemia.

*The concentration of iron in sow's milk*

Samples of milk were collected from eleven sows at different stages of lactation. They were weighed, dried in vacuo at – 15 to – 25° C. and the weight of solid matter determined. The dried material was ground up and duplicate samples were weighed out into clean silica crucibles, ashed at 450° C., and the ash dissolved in HCl, care being taken throughout to avoid iron contamination. Iron was determined with thiocyanate, by a method usually used for the assay of serum iron (McCance, Widdowson and Edgecombe, 1943).

Table IX shows the concentration of iron per 100 g. of liquid milk from sows who had and had not received supplementary iron. Each figure is the average of at least two samples. It will be seen that the feeding of iron to the sow during pregnancy or lactation made no difference to the concentration of iron in her milk. Colostrum tended to be richer in iron than milk secreted subsequently. From Tables VII and IX a calculation may be made of the approximate amounts of iron that the piglets may have ingested from their mother's milk. If it is assumed that during the first week the piglets feed 12 times a day, and on each occasion take about 35 g. containing 0·2 mg. of iron per 100 g., then each piglet may have obtained 0·84 mg. of iron a day, or 5·9 mg. in the course of the week. During the next 14 days the milk may supply about 1·2 mg. of iron per day, or, say, a total of 23 mg. in the first three weeks of life. In order to grow at a reasonably normal rate, and at the same time to maintain its haemoglobin level, a piglet requires to absorb and retain between

250 to 350 mg. of iron during this time. It will be recalled that, even in piglets reared in clean concrete sties, the amount of iron in their bodies increased by some 78 mg. in their first three weeks, so that they must have obtained the major part from their surroundings : had they not done so they would almost certainly have died. A trough of soil or sods inside the pen still seems to be the best solution of the problem.

TABLE IX

THE CONCENTRATION OF IRON IN SOW'S MILK (μG. PER 100 G.)

| | *Medication of sow* | | |
|---|---|---|---|
| | *No iron* | *Iron during pregnancy* | *Iron during pregnancy and lactation* |
| Colostrum ... | 265 | 235 | 282 |
| One day ... ... | 215 | 125 | 137 |
| Five to seven days | 179 | 153 | — |
| Ten to twelve days | 180 | 240 | 181 |

SUMMARY

The administration of iron to sows during pregnancy did not materially alter the size or composition of their offspring.

The administration of iron to lactating sows did not raise the percentage of iron in their milk.

It has been found in these experiments that a piglet contains about 50 mg. of iron at birth ; a piglet must retain about 7 mg. of iron per day to grow at a normal rate without becoming anaemic ; the milk of the sow cannot be expected to supply more than 1 mg. per day ; even in concrete sties piglets may retain 4 mg. of iron per day.

The amounts of iron found in the liver at birth were insufficient to make any serious contribution to the requirements of the body during the three following weeks.

The administration of iron to piglets during the first three weeks of life made them take more milk and grow more quickly, and greatly increased the amounts of iron found in the blood, in the liver and in the rest of the body.

The body of a piglet, reared outside with free access to soil, contained more iron than the bodies of those which had been given iron medicinally. The soil was found to contain 1·5 per cent. of iron, and the digestive tract of the piglet contained 0·5 g.

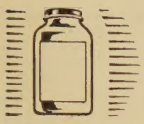
ACKNOWLEDGMENT

This work was made possible by a grant to one of us (J. A. J. V.) by the Agricultural Research Council. Part of the expenses were defrayed by the Medical Research Council. The authors are very grateful to Miss C. M. Walsham for the assistance she has given them throughout the whole of the investigation.

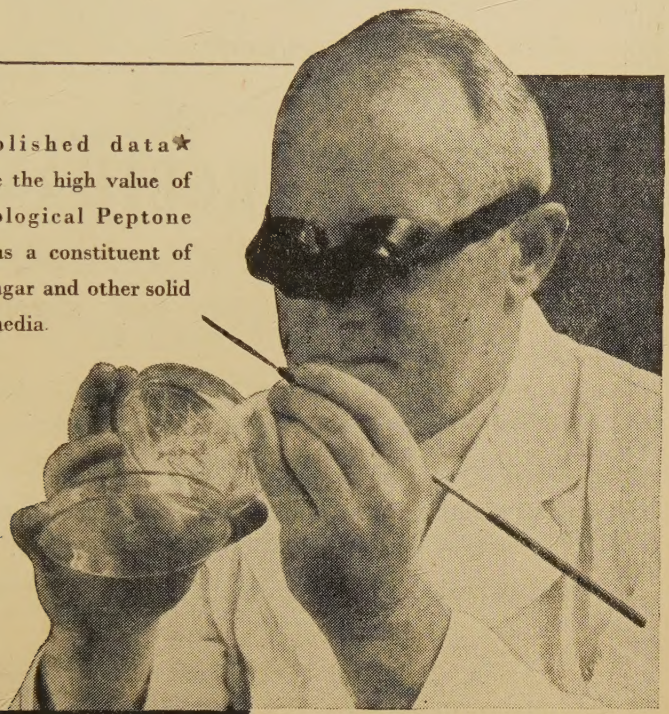
REFERENCES

Baskett, R. G., and Lamont, H. G. (1936). *Agric. Prog.*, **13,** 93.
Braude, R., and Foot, A. S. (1946). *Vet. J.*, **102,** 71.
Courtice, F. C. (1943). *J. Physiol.*, **102,** 441.
Doyle, L. P. (1932). *J. Amer. vet. med. Ass.*, **80,** 356.
Doyle, L. P., Mathews, F. P., and Whiting, R. A. (1928). *Ibid.*, **72,** 491.
Elvehjem, C. A. and Hart, E. B. (1932). *J. biol. Chem.*, **95,** 636.
Fargo, J. M. (1936). *Wiscon. Agric. exp. Sta. Bull.* No. 435.
Fulton, J. S. (1932). *Vet. Med.*, **27,** 103.
Hamilton, T. S., Hunt, G. E., Mitchell, H. H., and Carroll, W. E. (1930). *J. agric. Res.*, **40,** 927.
Hart, E. B., Elvehjem, C. A., Steenbock, H., Kemmerer, A. R., Bohstedt, G., and Fargo, J. M. (1929–30). *J. Nutrit.*, **2,** 277.
Hill, R. (1930). *Proc. roy. Soc. B.*, **107,** 205.
Houk, A. E. H., Thomas, A. W., and Sherman, H. C. (1946). *J. Nutrit.*, **31,** 609.
Kernkamp, H. C. H. (1935). *J. Amer. vet. med. Ass.*, **87,** 37; (1941). *Ibid.*, **99,** 373.
Lintzel, E., Rechenberger, J., and Shairer, E. (1944). *Ztschr. ges. exper. Med.*, **113,** 591.
McCance, R. A., Widdowson, E. M., and Edgecombe, C. N. (1943). *Lancet*, **2,** 126.
McCance, R. A., Widdowson, E. M., and Shackleton, L. R. B. (1936). *Med. Res. Coun. Spec. Rept. Ser. No.* 213.
McGowan, J. P., and Crichton, A. (1923). *Biochem. J.*, **17,** 204; (1924,a). *Ibid.*, **18,** 265; (1924,b). *J. Path. Bact.*, **27,** 201.
Moe, L. H., Craft, W. A., and Thompson, C. (1935). *J. Amer. vet. med. Ass.*, **87,** 302.
Schofield, F. W. (1931). *Rep. Ont. vet. Coll. for* 1930, p. 57.
Shackleton, L., and McCance, R. A. (1936). *Biochem. J.*, **30,** 582.
Schültze, M. O., Elvehjem, C. A., and Hart, E. B. (1936). *J. biol. Chem.*, **116,** 93.
Venn, J. A. J. (1944). *Nature*, **153,** 591.

[*Received for publication June 21st, 1947.*]



# An established product for the preparation of solid culture media



**P**ublished data★ emphasise the high value of Bacteriological Peptone (Evans) as a constituent of nutrient agar and other solid culture media.

**EVANS**
**BACTERIOLOGICAL**
**PEPTONE**

| | | | |
|---|---|---|---|
| ★ *Lancet* | 1941, | **1,** | 175 |
| ,, | 1941, | **2,** | 268 |
| *J. Path. Bact.* | 1942, | **54,** | 138 |
| ,, | 1942, | **54,** | 193 |
| ,, | 1944, | **56,** | 377 |
| *Brit. med. J.* | 1943, | **1,** | 377 |
| *Mthly. Bull. Inst. Med. Tech.* | 1944, | **10,** | 19 |

**Further details sent on request**

*Made in England by*

## EVANS MEDICAL SUPPLIES LTD

**Liverpool and London**

94-9/K7

OVERSEAS COMPANIES AND BRANCHES:
AUSTRALIA, BRAZIL, CHINA, EIRE, INDIA, MALAYA, PALESTINE, SOUTH AFRICA

'AVLON'

*brand*

# HEXACHLOROETHANE

## For the treatment of Liver Fluke infestation in cattle and sheep

Carbon tetrachloride, although well established in the treatment of fascioliasis in sheep, is generally regarded as being too toxic for use in cattle.

Hexachloroethane, while it is at least as effective as carbon tetrachloride is far less toxic, and can be regarded as the treatment of choice for cattle as well as sheep.

'Avlon' brand of Hexachloroethane dispersible powder is of the highest purity and is specially prepared for administration as a drench.

In districts where heavy fluke infestation is known to exist routine dosing of all susceptible animals with Hexachloroethane is recommended.

*Available in tins of 1 lb. and 7 lbs.*

**IMPERIAL CHEMICAL [PHARMACEUTICALS] LIMITED**
**THE RIDGE, BEECHFIELD ROAD, ALDERLEY EDGE, MANCHESTER**

Ph.184b

MANUFACTURERS OF

**Microscopes and all allied Apparatus**

**Photographic Lenses, Cameras, Etc.**

**Optical Elements for all purposes**

★ For many years Watsons have been pioneers in microscope design. Considerable development work is taking place in their Research Department. Is your name on the mailing list to receive bulletins when published, giving particulars of new apparatus and accessories?

# W. WATSON & SONS, LTD.

*Established 1837*

313, HIGH HOLBORN, LONDON, W.C.1

TELEPHONE: HOLB. 2767/8 TELEGRAMS: OPTICS HOLB. LONDON

# SULPHONAMIDE THERAPY

May & Baker Ltd. offer the following sulphonamides (and preparations thereof) for use in veterinary practice:

## PARENTERAL

**'M&B 683 SOLUBLE'** brand sulphapyridine soluble.

**'SOLUSEPTASINE'** brand solucin, neutral soluble sulphanilamide derivative.

**'SOLUTHIAZOLE'** brand neutral soluble sulphathiazole derivative.

**'SOLUPYRIDINE'** brand neutral soluble sulphapyridine derivative.

VETERINARY PRODUCTS M&B TO THE PROFESSION ONLY

*manufactured by*

**MAY & BAKER LTD.**

## ORAL

**SULPHANILAMIDE—M&B**

**'M&B 693'** brand sulphapyridine.

**'THIAZAMIDE'** brand sulphathiazole.

## ORAL—bowel active

**'THALAZOLE'** brand phthalylsulphathiazole.

## TOPICAL

**SULPHANILAMIDE—M&B**

**'THIAZAMIDE'** brand sulphathiazole.

**'THIAZAMIDE'** with Proflavine Hemisulphate 1 per cent.

**'M&B 693'** brand sulphapyridine.

*Further information and complete technical data are available on request from our Veterinary Department.*

DISTRIBUTORS

**PHARMACEUTICAL SPECIALITIES (MAY & BAKER) LTD., DAGENHAM**

47131

# H. K. LEWIS & Co. Ltd.

**MEDICAL BOOKSELLERS and PUBLISHERS**

## BOOKSELLING DEPARTMENT

Large stock of BOOKS ON MEDICINE, SURGERY AND ALL BRANCHES OF SCIENCE. FOREIGN DEPARTMENT.: Select stock available. Books not in stock obtained under licence.

OSTEOLOGY, ANATOMICAL DIAGRAMS, WALL CHARTS, ETC.

## SECOND-HAND DEPARTMENT 140 GOWER STREET

Large stock of second-hand recent editions.

**STATIONERY DEPARTMENT.** Medical and Scientific Stationery.

## LENDING LIBRARY Medical and Scientific

ANNUAL SUBSCRIPTION FROM ONE GUINEA.

Detailed Prospectus Post Free on Application.

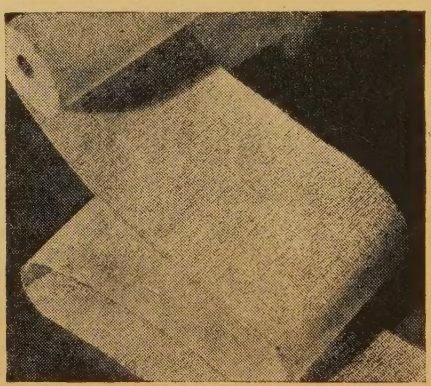
Bi-monthly List of New Books and New Editions Post Free to any address regularly. The Library Catalogue, revised to December, 1943, containing classified Index of Authors and Subjects. To subscribers, 12s. 6d. net; to Non-Subscribers, 25s. net; postage 8d. Supplement 1944 to December 1946. To Subscribers 2s. 6d. net; postage 4d. To Non-Subscribers 5s. net; postage 4d.

PLEASE STATE INTERESTS WHEN WRITING

**136 GOWER ST., LONDON W.C.1**

Telephone: EUSton 4282

Telegrams: Publicavit, Westcent, London



The even impregnation and perfect finish of the Gypsona Plaster of Paris Bandage ensure a light cast of great strength.

# Gypsona

TRADE MARK

**PLASTER OF PARIS BANDAGES, WIDE MATERIAL AND SLABS**

Made in England by

T. J. Smith & Nephew Ltd., Hull.